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<b>(21) International Application Number: PCT/US90/01031</b> <b>(22) International Filing Date: 23 February 1990 (23.02.90)</b>  <b>(30) Priority data:</b> 316,992                      24 February 1989 (24.02.89)    US 482,259                      16 February 1990 (16.02.90)    US  <b>(71) Applicant: IMMUNOTHERAPEUTICS, INC. [US/US];</b> 509 25th Avenue North, Fargo, ND 58102 (US).  <b>(72) Inventors: VOSIKA, Gerald, J. ; 3505 Riverview Circle,</b> Moorhead, MN 56560 (US). <b>CORNELIUS, Dennis, A. ;</b> 516 First Street, Fargo, ND 58102 (US). <b>GILBERT,</b> Carl, W. ; 1634 30th Avenue South, Fargo, ND 58103 (US).	<b>(74) Agent: SCHUMANN, Michael, D.; Merchant, Gould,</b> Smith, Edell, Welter & Schmidt, 3100 Norwest Center, 90 South Seventh Street, Minneapolis, MN 55402 (US).  <b>(81) Designated States: AT (European patent), BE (European</b> patent), CA, CH (European patent), DE (European pa- tent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European pa- tent), NO, SE (European patent).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>	
<b>(54) Title: IMMOBILIZED CYTOKINES</b>  <b>(57) Abstract</b>  <p>The present invention provides immobilized cytokines comprising cytokines bound to a solid, preferably biologically inert, support. The bound cytokine, for example IL-2, retains comparable biological activity when bound to the support. Accordingly, the bound cytokine is able to be utilized repeatedly and/or in significantly smaller quantities, as compared to an individual soluble cytokine. Cytokines of the present invention include, but are not limited to, IL-1-alpha, IL-1 beta, rIL-2, IL-2, IL-3, IL-4, IL-6, MuGMCSF, HuGMCSF, HuGCSF, HuEPO, <i>alpha</i>-interferon, <i>gamma</i>-interferon, TNF-alpha, HuILGF-I, HuILGF-II, FGFb, TGF-beta-II, HuEGF, HuPDGF.</p>		

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WO 90/09798

PCT/US90/01031

1  
**IMMOBILIZED CYTOKINES**

**Technical Field**

The present invention relates to cytokines that  
5 are immobilized on a solid support.

**Background of the Invention**

A number of biologically active mediators,  
generally termed cytokines, are produced by a variety of  
10 cells. Cytokines are produced on an obligatory basis  
for the maintenance of normal homeostasis, and also in  
response to pathological stimuli, such as immunological,  
infectious, and inflammatory processes. Those cytokines  
first described as products of lymphocytes are often  
15 referred to as "lymphokines," and those cytokines  
originally described as products of monocytes have been  
termed "monokines." Certain cytokines are also referred  
to as growth factors or colony stimulating factors,  
based on their effect on cell growth.

20 Examples of cytokines include: the lymphokines  
interleukin-1 (IL-1), interleukin-2 (IL-2), and  
interleukin-3 (IL-3); the monokine gamma interferon; and  
the growth factors granulocyte-macrophage colony  
stimulating factor (GMCSF) and erythropoietin (EPO).

25 Various cytokines serve as endogenous  
regulators (autocrines) and/or as intercellular  
signals. Many of these cytokines, initially recognized  
by a single biological activity, have been shown to have  
multiple, overlapping biological activities, often  
30 acting synergistically to amplify the biological  
response. The ultimate effect on the target cell  
includes regulation of growth, mobility,  
differentiation, and/or protein synthesis.

Interleukin-1 (IL-1), also known as lymphocyte  
35 activating factor, is produced by human monocytes,  
lymphocytes, endothelial cells, and fibroblasts. IL-1  
promotes lymphocyte differentiation, as indicated by  
changes in phenotypic cell surface markers. In  
addition, IL-1 stimulates T-lymphocyte functions and

WO 90/09798

PCT/US90/01031

2

increases the production of lymphokines such as IL-2, colony-stimulating factors (CSF), B-cell growth factor (BCGF), gamma-interferon ( $\gamma$ -IFN), and lymphocyte-derived chemotactic factors (LDCF), each with their own

5 biological effects. IL-1 also augments the in vitro proliferation, differentiation, and antibody-producing functions of B-lymphocytes. These and other biological activities have made IL-1 a valuable lymphokine in a wide variety of in vivo and in vitro uses.

10 Interleukin-2 (IL-2) was first termed T-cell growth factor (TCGF) for its ability to induce T-lymphocytes to proliferate and enable normal T-lymphocytes to be maintained continuously in culture. Like IL-1, IL-2 has been found useful in a wide variety of in vivo and in vitro applications. IL-2, when used as a vaccine adjuvant, overcomes genetic nonresponsiveness to malaria sporozoite peptides and enhances protection against Herpes simplex and rabies viruses. See M. F. Good et al., J. Immunol., 141, 972 (1988) and  
15  
20 A. Weinberg et al., J. Immunol., 140, 294 (1988).

Among its biological activities when used as a pharmacological agent, in vitro IL-2 results in the proliferation and differentiation of a group of more selective T-cell populations known as lymphokine  
25 activated killer cells, tumor infiltrating lymphocytes, and cytotoxic T-cells. Such cells have been demonstrated in vitro to be cytotoxic to allogenic normal target cells and to both immunogenic and nonimmunogenic tumor cells. See S. A. Rosenberg, J. Nat. Can. Inst.,  
30 75, 595 (1985); S. A. Rosenberg, J. Immunol., 121, 1951 (1978); and S. A. Rosenberg et al., Science, 233, 1318 (1986).

In vitro lymphokine activated killer cells have been used in combination with the in vivo administration  
35 of interleukin-2 to achieve an improved antitumor effect. The infusion of in vitro IL-2 activated killer cells and the concurrent administration of IL-2 has



WO 90/09798

PCT/US90/01031

3

demonstrated antitumor activity in both animals and humans; such activity generally exceeding that observed with the use of IL-2 or lymphokine activated killer cells individually. See J. J. Mule et al., Science, 5 225, 1487 (1984); R. Lafrenier, and S. A. Rosenberg, Cancer Res., 45, 3735 (1985); S. A. Rosenberg et al., N. Engl. J. Med., 316, 889 (1987); J. J. Mule et al., J. Immunol., 136, 3899 (1986); H. W. West et al., N. Engl. J. Med., 316, 898 (1987); S. A. Rosenberg et 10 al., N. Engl. J. Med., 313, 1485 (1985).

The growth of tumor infiltrating lymphocytes obtained from human malignancies has been induced by interleukin-2, in vitro, for periods of up to 60 days. These lymphocytes have demonstrated human antitumor 15 activity in patients with lung cancer when administered without the concurrent intravenous administration of interleukin-2. See R. L. Kradin et al., Can. Immunol. Immunother., 24, 76 (1987).

Additional cytokines synthesized by T-cells 20 include migration inhibition factor (inhibits the random migration of macrophages); leukocyte inhibition factor (inhibits the random migration of neutrophils); macrophage activation factor (enhances the cytolytic activity of macrophages); fibroblast activation factor 25 (stimulates proliferation of fibroblasts); and interleukin-3 (IL-3) (activity similar to colony-stimulating factor).

Although the mechanistic details for cytokine activity are not known with certainty, the general 30 mechanism for activity is believed to include the steps of: 1) binding of the cytokine to a specific cell surface receptor; 2) initiation of certain "cell surface activated" events; and 3) internalization of the cytokine-receptor complex where internal interactions 35 result in proliferation, growth, differentiation, and/or the expression of specialized cell function.

Specifically, in the case of IL-2, the

WO 90/09798

PCT/US90/01031

4

interaction of IL-2 with T-cells is believed to involve an initial interaction with a low affinity receptor, IL2Rb, resulting in induction of a second receptor molecule, IL2Ra, that forms a high affinity complex with IL-2. Association of IL-2 with the high affinity complex results in proliferation. In this process of activation and proliferation there is an internalization of the IL-2-receptor complex and a subsequent decrease in the number of surface IL-2 receptors. See K. A. Smith, Science, 240, 1169 (1988).

It has been suggested that cell surface associated events are sufficient for activity and that internalization of the receptor-ligand complex is not required, at least in certain cases. Porcine insulin and murine alpha/beta interferon bound to Sepharose via cyanogen bromide activation have been reported to have biological activity, through cell surface associated events. See P. Cuatrecasas, Proc. Nat. Acad. Sci. USA, 63, 450 (1969); H. Ankel et al., Proc. Nat. Acad. Sci. USA, 70, 2360 (1973); and C. Chaney et al., Proc. Soc. Exp. Biol. Med., 147, 293 (1974).

The accuracy of these reports has been doubted by persons in the art, particularly because of the known instability of the particular covalent bond formed. See W. H. Scouten, Methods in Enzymology, Klaus Mosbach, ed., Academic Press Pub., 135, 31 (1987). The question of the necessity of internalization has remained a debated issue. See E. DeMaeyer and J. DeMaeyer-Guignand, Interferon and Other Regulatory Cytokines, John Wiley and Sons Pub., 67-90 (1988).

The cost, availability, and toxicity of cytokines, such as IL-2, can be a limiting factor in the usefulness of the cytokine as a biologically active agent. Therefore, it would be desirable to be able to reuse and/or use less of a particular cytokine while retaining a substantial amount of their biological activity, with possibly decreased toxicity.

WO 90/09798

PCT/US90/01031

5

Therefore, a continuing need exists for modified cytokines that retain comparable, and in some cases, improved biological activity when compared with corresponding soluble or free cytokines, thus providing  
5 a biologically active cytokine that can be reused to stimulate biological activity and/or that can be used in significantly smaller quantities.

#### Brief Description of the Invention

10 The present invention provides immobilized cytokines comprising cytokines firmly bound to a solid, preferably biologically compatible, insoluble immobilizing support. The bound cytokine retains substantially the activity of the free cytokine when  
15 bound to the support. Accordingly, the bound cytokine is able to be utilized repeatedly (reused) to stimulate biological activity, and/or used in significantly smaller total quantities than the corresponding soluble or free cytokine.

20 Cytokines useful in the present invention include, but are not limited to, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, tumor necrosis factor (TNF), gamma-interferon, alpha-interferon, beta-interferon, erythropoietin (EPO), granulocyte colony stimulating  
25 factor (GCSF), murine granulocyte colony stimulating factor (MuGCSF), granulocyte-macrophage colony stimulating factor (GMCSF), murine granulocyte-macrophage colony stimulating factor (MuGMCSF), insulin-like growth factor I (ILGF-I), insulin-like growth  
30 factor II (ILGF-II), transformation growth factor beta (TGF- $\beta$ ), epidermoid growth factor (EGF), platelet derived growth factor (PDGF), and fibroblast growth factor-basic (FGFb). Preferred cytokines include those described in the Examples, and more preferably IL-2,  
35 GMCSF, GCSF, EPO, TNF, FGFb, TGFb, EGF, and PDGF.

The cytokine is preferably bound to a biologically compatible, particulate support by means of

WO 90/09798

PCT/US90/01031

6

covalent bonding, preferably by means of a linking arm. The cytokine is preferably firmly bound to the support in a manner that allows the activity of the cytokine to be stabilized by the immobilization. The activity thus  
5 is durable and reusable.

As used herein, "substantially the activity of the free cytokine" means that at least one of the one or more active sites of a cytokine remains active, and will produce significant biological activity as a bound  
10 cytokine. In other words, because cytokines have multiple, often overlapping, biological or regulatory effects, a bound cytokine of the present invention may demonstrate one or more activities the same as, or similar to, that of the free cytokine. Thus, in  
15 demonstrating efficiency of an immobilized cytokine of the present invention, one or more biological activities may be stabilized through immobilization. Thus, at least one activity is preserved in the bound state, and in some cases may be enhanced by the binding of the  
20 cytokine to the support.

The structure or length of the linking arm may be varied to optimize the biological activity of the bound cytokine. Preferred linking arms comprise one or more linking groups selected from the group  
25 consisting of: (a) diamines, having the general formula  $\text{NH}_2\text{-R}^1\text{-NH}_2$ , where  $\text{R}^1$  is a  $\text{C}_2\text{-C}_{20}$  alkyl group; (b) amino acids, having the general formula  $\text{NH}_2\text{-R}^2\text{-CO}_2\text{H}$ , where  $\text{R}^2$  is a  $\text{C}_1\text{-C}_{20}$  alkyl group; and (c) dialdehydes, having the general formula  $\text{OHC-R}^3\text{-CHO}$ , where  $\text{R}^3$  is a  $\text{C}_1\text{-C}_{20}$  alkyl  
30 group.

Useful supports include, but are not limited to, fibers, microspheres, beads, particles, membranes, sheets, and the like.

As used herein, "cytokine" refers to the  
35 natural or recombinant form of the cytokine, as well as to modified sequences, biologically active fragments or portions of cytokines, genetically or chemically

WO 90/09798

PCT/US90/01031

7

modified forms of a cytokine, biologically equivalent synthetic ligands, or mixtures thereof, which exhibit a substantially equivalent profile of bioactivity, or a portion of the original profile of bioactivity.

5           The present invention also provides methods of using the immobilized cytokines, both in vitro and in vivo, for the proliferation, growth, differentiation and/or expression of specialized cell function, including inducing growth of a cytokine-dependent cell  
10 line, such as an IL-2 dependent cell line, by contacting it with an effective amount of an immobilized cytokine of the invention.

#### Summary of the Drawings

15           Fig. 1 is a graphical depiction of the growth of CTLL-2 cells (DPM's  $\times 10^{-3}$ ) using IL-2 immobilized via a carboxyl group of the IL-2 as compared to IL-2 immobilized via an amino group of the IL-2.

20           Fig. 2 is a graphical depiction of the concentration dependence of immobilized IL-2 ( $\mu\text{g}$  IL-2 in initial coupling reaction) on the growth of CTLL-2 cells, a cytotoxic T-lymphocyte cell line, as determined by [ $^3\text{H}$ ]-thymidine incorporation (DPM's  $\times 10^{-3}$ ).

25           Fig. 3 is a graphical depiction of the growth of CTLL-2 cells (DMP's  $\times 10^{-3}$ ) using immobilized IL-2 as a function of time (hours) relative to the growth of CTLL-2 cells using soluble IL-2.

30           Fig. 4 is a graphical depiction of the growth of human peripheral blood lymphocytes (PBL's in DPM's  $\times 10^{-3}$ ), using immobilized IL-2 as a function of time (hours) relative to the growth of PBL's using soluble IL-2.

35           Figs. 5A and 5B are graphical depictions of stimulation of granulopoiesis as measured by an increase in the white blood count of mice receiving soluble (5A) or immobilized (5B) MuGMCSF.

          Fig. 6 is a graphical depiction of stimulation

WO 90/09798

PCT/US90/01031

8

of granulopoiesis as measured by the increase in white blood count of cyclophosphamide-treated mice receiving soluble or immobilized recombinant murine GMCSF (rMuGMCSF).

- 5           Fig. 7 is a graphical depiction of the stability of covalent bound rMuGMCSF as measured by its retention in contrast to adsorbed rMuGMCSF following SDS washes.

10           Detailed Description of the Invention  
          Cytokines

- Interleukin-2 (IL-2) is commercially available as T-cell growth factor (human interleukin-2 recombinant; T3267) and as derived from cultured rat  
15   splenocytes (TD892) from Sigma Chemical Co., St. Louis, MO. Recombinant IL-2 (ala-125 analog and natural sequence) is also commercially available from Amgen, Thousand Oaks, CA. Natural sequence recombinant  
interleukin-3 (IL-3), natural sequence recombinant  
20   interleukin-4 (IL-4), and natural sequence recombinant interleukin-6 (IL-6) are commercially available from Amgen, Thousand Oaks, CA.

- Recombinant human granulocyte-macrophage colony stimulating factor (rHuGMCSF), recombinant human  
25   granulocyte colony stimulating factor (rHuGCSF), recombinant human erythropoietin (rHuEPO), recombinant murine granulocyte-macrophage colony stimulating factor (rMuGMCSF), recombinant human gamma interferon (rHuIFN-gamma), and recombinant human epidermoid growth  
30   factor (rHuEGF) and fibroblast growth factor-basic (FGFb) all are available from Amgen, Thousand Oaks, CA. Recombinant human platelet derived growth factor (rHuPDGF), recombinant human insulin-like growth factor I (rHuILGF-I), recombinant human insulin-like growth  
35   factor II (rHuILGF-II), and transformation growth factor alpha (TGF-alpha) are commercially available from Bachem, Torrance, CA. Transformation growth factor

WO 90/09798

PCT/US90/01031

9

beta, porcine, (pTGF-beta) is commercially available from R & D Systems, Minneapolis, MN. Transformation growth factor beta is also commercially available from Collaborative Research, Bedford, MA. Recombinant  
5 interferon alpha is commercially available as Roferon™ from Roche Laboratories.

The biologically active portions of certain cytokines have also been isolated. The present invention also includes binding the biologically active  
10 portions of cytokines to a suitable support.

IL-1 has multiple effects on lymphocyte populations, including its function as an autocrine growth factor for many T-cell clones. IL-1 is also a potent stimulator of thymocyte proliferation, and of  
15 mitogen, nominal antigen plus Ia antigen, or alloantigen stimulated helper T-cells. IL-1 increases interleukin-2 receptor expression and IL-2 secretion of human peripheral T-cells in the presence of monoclonal antibody to the antigen-receptor complex. In addition,  
20 IL-1 acts as a cofactor for Con A activation of resting T-cells and is required for the proliferation of lymphocytes which express a high affinity receptor for IL-1. IL-1 is produced by human lung endothelium where it is believed to function in the development of  
25 inflammatory infiltrates.

IL-1 is also a regulator of hematopoietic activity. IL-1 induces endothelial cells to release granulocyte-macrophage colony stimulating factor (GMCSF) and granulocyte colony stimulating factor (GCSF), thus  
30 providing the mechanism by which IL-1 modulates granulocyte production and function during inflammation. IL-1 also releases GMCSF from monocytes and enhances growth factor dependent proliferation of human hematopoietic precursors.

35 IL-1 has demonstrated by its antitumor activity causing complete regression of relatively large immunogenic murine sarcomas by augmenting an ongoing

WO 90/09798

PCT/US90/01031

10

T-cell response. IL-1 has a direct cytotoxic effect in vitro on human A375 melanoma cells. IL-1 has also shown synergistic effects with interleukin-2 (IL-2) in the production of lymphokine activated killer cells.

5 This broad range of activity of IL-1 as a regulator of lymphocyte function, hematopoiesis, and lymphocyte antitumor activity has made IL-1 a valuable cytokine in a wide variety of in vivo and in vitro uses. See, for example, T. Hoang et al., J. Exp. Med.,  
10 168, 463 (1988); R. J. North et al., J. Exp. Med., 168, 2031 (1988); B. Tartakovsky et al., J. Immunol., 141, 3863 (1988); A. H. Lichtman et al., Proc. Nat. Acad. Sci. USA, 85, 9699 (1988); B. S. Bochner et al., J. Immunol., 139, 2297 (1987); and V.  
15 C. Broudy et al., J. Immunol., 139, 464 (1987).

Interleukin-3 (IL-3), also known as multicolony stem cell activating factor, or as multicolony stimulating factor, is a glycoprotein hematopoietic growth factor. IL-3 has a broad range of activity, due  
20 to its ability to stimulate both early stem cells, common to many myeloid cell lineages, as well as committed cells. IL-3 binds to a 140 kilodalton cell surface phosphoprotein. In primates, continuous infusion of IL-3 results in a delayed, modest increase  
25 in the white cell count. However, IL-3 has a marked synergistic effect on the response to subsequent treatment with low doses of granulocyte-macrophage colony stimulating factor, suggesting that IL-3 acts on early lineage cells that require a subsequent second  
30 factor to complete development. This hypothesis is consistent with tissue culture studies indicating that IL-3 is more effective in supporting colony formation by blast cells. In addition, IL-3 itself will not support  
in vitro colony formation, but requires a later acting  
35 factor, such as GMCSF. IL-3 acts synergistically with IL-6 to support early blast colony formation, with granulocyte colony stimulating factor (GCSF) to enhance



WO 90/09798

PCT/US90/01031

11

neutrophil formation, and with GMCSF to enhance granulocyte and macrophage colony formation. This broad range of activity as a hematopoietic cytokine makes IL-3 a valuable adjunct to hematopoietic cytokine therapy.

5           Like other cytokines, IL-3 also has negative regulatory effects, as evidenced by its inhibition of lymphokine activated killer cells. To date, IL-3 has been the only cytokine implicated in the regulation of early B-cell development, as is evidenced by the IL-3  
10 dependent clones from murine fetal liver or adult bone marrow which show characteristics of B-cell precursors. See, for example, R. E. Donahue et al., Science, 241, 1820 (1988); R. J. Isfort et al., Proc. Nat. Acad. Sci. USA, 85, 7982 (1988); D. Rennick et al., J. Immunol.,  
15 142, 161 (1989); and G. Gallagher et al., Clin. Exp. Immunol., 74, 166 (1988).

Interleukin-4 (IL-4) is also known as B-cell stimulatory factor-1 (BSF-1), B-cell differentiation factor (BCDF), and B-cell growth factor 1 (BCGF-1). In  
20 the murine system, IL-4 enhances immunoglobulin IgG1 and IgE production in lipopolysaccharide activated cells, increases the expression of histocompatibility antigens on B-cells, and is required for the proliferation of anti-IgM activated B-cells.

25           In human studies a similar effect to that observed in the murine system on lymphocyte function has been described. High affinity receptors exist for IL-4 on both human hematopoietic and nonhematopoietic cells. IL-4 can induce proliferation in unstimulated  
30 thymocytes, and the response is strongly augmented with mitogens. IL-4 also augments the mitogen induced stimulation of human peripheral T-cells in the presence of dexamethasone, which inhibits IL-2 production. IL-4 also down modulates IL-2 induced human B-cell  
35 proliferation, and inhibits IL-2 induced NK cell activation and proliferation. IL-4, in conjunction with IL-2, however, augments the growth of tumor infiltrating

WO 90/09798

PCT/US90/01031

12

lymphocytes to autologous human malignant melanoma. In addition to its effects on lymphoid cells, IL-4 interacts with GMCSF and EPO to enhance granulocyte-macrophage and erythroid cell forming units. See, for example, H. Spits et al., J. Immunol., 139, 1142 (1987); Y. Kawakami et al., J. Exp. Med., 168, 2183 (1988); A. Nagler et al., J. Immunol., 141, 2349 (1988); A. Vazquez et al., J. Immunol., 142, 94 (1989); T. DeFrance et al., J. Exp. Med., 168, 1321 (1988); S. Karray et al., J. Exp. Med., 168, 85 (1988); and B. Brooks and R. C. Rees, Clin. Exp. Immunol., 74, 162 (1988).

Interleukin-6 (IL-6) is also known as B-cell stimulatory factor-2, interferon beta-2, and hybridoma-plasmacytoma growth factor. IL-6 is a multi-functional cytokine, initially described as a T-cell lymphokine with antiviral activity. IL-6 has been demonstrated to be produced by a variety of cells including: monocytes, fibroblasts, hepatocytes, cardiac myxomas, brain glial cells, and vascular endothelium. IL-6 activity is believed to include: regulation of fibroblast activity; acute phase protein production by hepatocytes; stimulation of human thymocytes and T-lymphocytes in the presence of mitogen; proliferation and differentiation of murine T-lymphocytes into cytotoxic cells; maintenance of myeloma derived cell lines; autocrine signalling for human multiple myeloma, and inhibition of the growth of carcinoma and leukemia/lymphoma cell lines. See, for example, P. B. Sehgal et al., Science, 235, 731 (1987); S. Shimizu et al., J. Exp. Med., 169, 339 (1989); J. L. Ceuppens et al., J. Immunol., 141, 3868 (1988); G. Tosato and S. E. Pike, J. Immunol., 141, 1556 (1988); M. Lotz et al., J. Exp. Med., 167, 1253 (1988); and L. Chen et al., Proc. Nat. Acad. Sci. USA, 85, 8037 (1988).

Granulocyte-macrophage colony stimulating

WO 90/09798

PCT/US90/01031

13

factor (GMCSF), granulocyte colony stimulating factor (GCSF), macrophage colony stimulating factor (MCSF) and multi-colony stimulating factor (IL-3) constitute a family of glycoproteins that have been recognized by  
5 their ability to stimulate and regulate the process of proliferation and differentiation of hematopoietic cells both in vivo and in vitro. These individual cytokines are produced by one or more of the following cell sources: T-lymphocytes, monocytes, fibroblasts,  
10 epithelial cells, or endothelial cells. Additionally, IL-1, also known as hemopoietin-1, participates in this regulatory network by enhancing the effects of IL-3, MCSF, GCSF, and GMCSF.

Studies in animals have shown that GMCSF, GCSF,  
15 and IL-3 increase the number of functional white blood cells, and that the effect is enhanced by IL-1. Sequential administration of IL-3 and GMCSF has resulted in an increased platelet count as well. Use of members of this group of cytokines in nonhuman primates has  
20 shown benefit in viral induced pancytopenia, chemotherapy, and irradiation therapy induced myelo-suppression, leukopenia following whole body irradiation or high dose cytotoxic chemotherapy, and autologous bone marrow transplant.

25 In man the administration of both GCSF and GMCSF results in a significant increase in neutrophils and neutrophils-eosinophils respectively, as well as an increase in bone marrow cellularity with immature cells appearing in the blood. Clinical side effects following  
30 the use of GMCSF in man have included fever, rash, myalgia, fatigue, gastrointestinal distress, thrombophlebitis, bone pain, pleuritis, pleural effusion, pericarditis, and pulmonary emboli. The only side effect noted with GCSF has been bone pain.

35 The demonstrated benefits of GCSF and GMCSF in man have included: restoration of hematopoiesis following myelo-suppressive cytotoxic chemotherapy;

WO 90/09798

PCT/US90/01031

14

accelerated granulocyte recovery and decreased incidence of infection in patients with autologous bone marrow transplants; and improved circulating white cell, hemoglobin, and platelet count in patients with  
5 myelodysplastic syndrome and aplastic anemia. Administration of GMCSF to patients with AIDS associated leukopenia resulted in a significant increase in granulocytes and monocytes without increased viral production.

10 In addition to the hematopoietic effects described above, GMCSF has been demonstrated in vivo to activate monocytes to a tumoricidal state suggesting another potential clinical application for this cytokine. GMCSF has also been demonstrated to stimulate  
15 the proliferation in vitro of osteogenic sarcoma cell lines, a breast cancer cell line, a simian virus SV-40 transformed bone marrow stromal cell line and normal bone marrow fibroblast precursors. See, for example: S. Vadhan-Raj et al., N. Engl. J. Med., 319, 1628  
20 (1988); J. E. Groopman et al., N. Engl. J. Med., 317, 593 (1987); K. H. Grabstein et al., Science, 232, 506 (1986); S. Dedhar et al., Proc. Nat. Acad. Sci. USA, 85, 9253 (1988); and A. A. Jakubowski et al., N. Engl. J. Med., 320, 38 (1989).

25 Erythropoietin (EPO) is the single cytokine required for the continued differentiation of the hematopoietic cells that produce mature red blood cells. In in vitro studies, the combination of IL-3, GMCSF or GCSF with EPO has been required for red cell  
30 production, suggesting that these cytokines are involved in the maintenance of the red cell precursor whereas EPO is required for the terminal differentiation and maturation. See, for example, J. Suda et al., Blood, 67, 1002 (1986).

35 Tumor Necrosis Factor (TNF), also known as a multi-functional cytokine produced by monocytes-macrophages, is a particularly important

WO 90/09798

PCT/US90/01031

15

mediator of inflammatory response. Two forms, TNF-alpha (Cachectin) and TNF-beta, exist. Among its effects TNF is a major factor in gram-negative endotoxin shock and induces a profound wasting (cachexia) syndrome in  
5 patients with cancer and chronic disease.

The range of activity includes stimulation of fibroblast growth, stimulation of osteoblast activity and bone reabsorption, promotion of angiogenesis, stimulation of collagenase and prostaglandin E<sub>2</sub> in  
10 synovial cells, and stimulation of procoagulant and platelet-activating factor in endothelial tissue.

TNF is an autocrine produced by macrophages. It functions as an immunomodulator, activating macrophages and increasing their ability to specifically  
15 recognize and kill malignant cells. TNF is chemotactic for macrophages, indicating its production at a site of inflammation both recruits and activates macrophages.

TNF participates in the cytokine network and induces the release of IL-1, GM-CSF, platelet derived  
20 growth factor, and beta-2 interferon.

The major potential therapeutic effect of TNF is its antitumor activity. TNF is the mediator of endotoxin induced tumor regression. TNF may be involved in the antitumor activity of IL-2, since IL-2 induces  
25 TNF in human peripheral blood monocytes. TNF, given systemically, induces regression of tumor in mice. The direct anti-proliferative and tumor cytotoxic effects of TNF and IL-1 are believed synergistic.

Initial clinical studies of TNF in man have  
30 included intravenous and intramuscular injections. Toxicities have included: fever, chills, fatigue, anorexia, hypotension, and tachycardia. Several minor tumor responses have been noted to date. See, for example, B. Sherry and A. Cerami, J. Cell Biol., 107,  
35 1269 (1988); J. J. Mule et al., Cancer Immunol. Immunother., 26, 202 (1988); Y. Ichinose et al., Cancer Immunol. Immunother., 27, 7 (1988);

WO 90/09798

PCT/US90/01031

16

P. B. Chapman et al., J. Clin. Onc., 5, 1942 (1987);  
H. H Bartsch et al., Mol. Biother., 1, 21 (1988); and  
T. Steinmetz et al., J. Biol. Resp. Mod., 7, 417 (1988).

Interferon (IFN) is a term originally assigned  
5 to a class of compounds discovered in the late 1950's  
with anti-viral activity. Originally, three classes of  
interferon were designated as alpha, beta, and gamma;  
designating their original identification and isolation  
from leukocytes, fibroblasts, and lymphoid cells,  
10 respectively. As of 1988, at least 24 nonallelic genes,  
coding for structurally related forms of alpha-  
interferon, had been described. These were divided into  
two subfamilies designated IFN-alpha I genes, which code  
for proteins of 165-166 amino acids, and IFN-alpha II  
15 genes, which code for protein of 172 amino acids. A  
single gene coding for what is commonly called  
"fibroblast interferon" has been fully characterized in  
humans. However, fibroblast can produce more than one  
form of interferon and the more correct term for  
20 fibroblast interferon is human interferon beta (HuIFN-  
beta). HuIFN-beta has about 40% amino acid homology  
with HuIFN-alpha. The human interferon gamma gene  
exists as a single copy with some individual to  
individual allelism or difference in single amino  
25 acids. Gamma-interferon has no homology to alpha- or  
beta-interferon.

Interferon, or virus-induced proteins with  
anti-viral activity, have been identified from  
representatives of all vertebral classes except  
30 amphibia. The biological activity of various IFN-alpha  
subtypes are relatively similar. The biological  
activity of INF-alpha and beta are also similar, but  
both differ from IFN-gamma. See E. DeMaeyer and J.  
DeMaeyer-Guignard, Interferon and Other Regulatory  
35 Cytokines, John Wiley and Sons, Pub., pp. 5-38 (1988).  
The major biological activities of alpha and beta  
interferon are: antiviral effects; induction of

WO 90/09798

PCT/US90/01031

17

monocytes to express major histocompatibility, complex class II antigens, and interleukin-1; antiproliferative effects; and regulation of human natural killer cell activity.

5 Interferon alpha and beta have an antitumor effect that involves a number of mechanisms including, among others, an antiproliferative effect, induction of differentiation, regulation of oncogene expression, and stimulation of the immune response.

10 The exact biological effects may vary with the particular structural forms of alpha-interferon and with the sensitivity of the assay cell line. It is also possible to observe both positive and negative regulation, as for example, human interferon alpha or  
15 beta may inhibit the maturation of monocytes to macrophages. See E. DeMaeyer and J. DeMaeyer-Guidnand in Interferon and Other Regulatory Cytokines, John Wiley and Sons, Pub., pp. 134-153 (1988).

A group of cytokines also termed growth factors  
20 have, among their biological activities, a positive or negative regulatory effect on wound healing and tissue repair including chemotactic activity, proliferation, growth and differentiation of epithelial cells and fibroblasts, stimulation of matrix formation and  
25 cartilage formation, and vascular formation (angiogenesis). A large number of biologically active proteins have been described within this area and have been classified on taxonomical principles into families and species based on their biological effects and amino  
30 acid sequence homology (as shown below in Table 1). Although this group of cytokines has been associated with tissue repair, they have other biological effects. In addition, other cytokines such as interleukin-1 and interleukin-3, which regulate immune responses, also  
35 have an effect on tissue repair.

Epidermal growth factor (EGF) is a key representative member of a family of structurally

WO 90/09798

PCT/US90/01031

18

related proteins including transformation growth factor (TGF) alpha, amphiregulin, and vaccinia growth factors. Human EGF was first isolated from urine and named urogastrone by its ability to inhibit gastric secretion

5 (H. Gregory, Nature, 257, 324 (1975)). Murine EGF, isolated from the salivary gland is mitogenic for a large number of cell types including epithelial, fibroblasts, and endothelial cells (S. Nakagawa et al., Differentiation, 29, 284 (1985)). It stimulates

10 precocious eyelid opening and tooth eruption in newborn mice (S. Cohen, J. B. Chem., 237, 1555 (1962)), and is chemotactic for epithelial cells (J. Blay and K. D. Brown, J. Cell Physiol., 124, 107 (1985)). EGF is synthesized as a precursor protein which is processed

15 into a 53-amino acid active protein.

Transformation growth factor alpha (TGF-alpha) binds to the same receptor as EGF and shares similar biological activity. See G. J. Todaro et al., Proc. Nat. Acad. Sci. USA, 77, 5258 (1980).

20 TGF-alpha, like EGF is synthesized as a 160-amino acid precursor, which is proteolytically processed into a 50-amino acid biologically active residue. See R. Derynck et.al., Cell, 38, 287 (1984). TGF-alpha was originally recognized by its ability to synergize with TGF-beta to

25 induce anchorage independent growth of normal rat kidney fibroblast. See M. A. Azano et al., Proc. Nat. Acad. Sci. USA, 80, 6264 (1983).

Platelet derived growth factor (PDGF) is purified from human blood platelets. See R. Ross and

30 A. Vogel, Cell, 14, 203 (1978). It consists of two polypeptide chains: the A chain (124 amino acid residues); and the B chain (140 amino acid residues). PDGF is a potent mitogen for cells of mesenchymal origin (e.g., smooth muscle and fibroblasts) but has no effect

35 on epithelial or endothelial cells which lack PDGF receptors. See R. Ross, E. W. Raines, and D. F. Bowen-Pope, Cell, 45, 155 (1986). Platelet



WO 90/09798

PCT/US90/01031

19

derived growth factor may also be obtained from porcine cells.

Transformation growth factor beta(s) were originally identified by their ability to act synergistically with EGF or TGF-alpha to induce anchorage independent growth of NRK cells. See M. A. Anzano et al., Proc. Nat. Acad. Sci. USA, 80, 6264 (1983). They have subsequently been shown to have multiple biological effects including, chemotaxis, mitogenesis, growth inhibition and the induction or inhibition of differentiation depending upon other growth factors present. See M. B. Sporn and A. B. Roberts et al., J. Cell Biol., 105, 1039 (1987). In their mature form, TGF-betas are acid and heat-stable disulfide-linked homodimeric proteins of 112 amino acid residues which share 70% homology. See R. Derynck and J. A. Farrett et al., Nature, 316, 701 (1985). Another member of the family, beta-3, has recently been described. See J. M. Wozney and V. Rosen et al., Science, 242, 1582 (1988).

Although they share a variety of biological activities, different forms of TGF also possess unique biological activities for select target cells. See F. Rosa and A. B. Roberts et al., Science, 239, 783 (1988). TGF-beta I has demonstrated major activity in wound healing. Other biologically active proteins included in the TGF-beta family include: forms of gonadal proteins designated inhibin and activin that regulate pituitary secretion of follicle stimulating hormone; Mullerian inhibiting substances that cause regression of the female Mullerian ducts in the developing male embryo; and bone morphogenic proteins that are a group of polypeptides involved in the induction of cartilage and bone formation. See J. M. Wozney and V. Rosen et al., Science, 242, 1528 (1988).

Fibroblast growth factors (FGF) are

WO 90/09798

PCT/US90/01031

20

single-chain proteins of 14-18 kilodaltons. Two well characterized forms are basic FGF, isolated from brain and pituitary, and acidic FGF, isolated from brain and retina. Basic FGF, in most systems, is more stable and has ten times the potency of acidic FGF. Both forms of FGF bind to the same receptor and are mitogenic for cells of mesodermal origin such as fibroblasts, vascular endothelial cells, vascular smooth muscle, myoblasts, chondrocytes and osteoblasts. See F. Esch and A. Baird et al., Proc. Natl. Acad. Sci. USA, 85, 6507 (1985).

The products of the int-2 and hst proto-oncogenes are also included as members of the FGF family. (See C. Dickson and P. E. Gordon, Nature, 326, 833 (1987).

Insulin-like growth factor I (ILG-I) also known as Somatomedin C, and Insulin-like growth factor II (ILG-II) represent a current nomenclature for a number of factors initially purified from serum and sharing the three biological activities of stimulating of sulfate incorporation into cartilage, insulin-like activity, and multiplication-stimulating activity. The liver and fibroblasts are major sources of circulating insulin-like growth factors, but essentially all tissues have been shown to produce them.

Insulin-like growth factors, among their biological activities, have also been shown to stimulate glucose metabolism, and stimulate DNA synthesis and cell proliferation of fibroblasts, sertoli cells, fetal brain cells, myoblasts, lens epithelium, pancreatic beta cells, lectin stimulating lymphocytes, and density arrested Balb/c 3T3 cells after being rendered "competent" with Platelet Derived Growth Factors. (See R. C. Baxter, Adv. Clin. Chem., 25, 50 (1986)).

Cytokines react with cell surface receptors which themselves are complex and may consist of subunits. Portions of the cytokine may bind preferentially to various subunits of the receptor resulting in different biological and/or regulatory

WO 90/09798

PCT/US90/01031

21

effects. The present invention also provides for immobilization of such cytokine fragments that may be directed to a particular subunit of the receptor.

#### 5 Immobilizing Supports

Support materials useful in the present invention are preferably biologically compatible, and may be nonbiodegradable or biodegradable as desired. It may be desirable that the support be biodegradable when  
10 the bound cytokine will be utilized in vivo, while insoluble support materials are useful in applications such as bioreactors.

Suitable supports include fibers, sheets, microspheres, particles, beads, membranes, and the like.

15 The support preferably comprises a surface which is chemically compatible with the covalent attachment of the cytokine. Accordingly, the support preferably includes a surface having appropriate functional groups which can covalently bind to a site on  
20 the cytokine (e.g., an amino or carboxyl site), or to a suitable linking arm that can bind to a site on the cytokine. If the intended support does not have suitable functional groups for cytokine binding, such groups can be provided by appropriate chemical  
25 modification of the support surface. For example, a nonfunctionalized polystyrene support can be provided with a functionalized surface by suitable functionalization of the aromatic rings (e.g., via bromination).

30 Not all binding chemistries work equally well with each of the many various cytokines. Suitability of a particular binding chemistry used may, in part, depend upon the availability of reactive sites, and their proximity to the active site of the cytokine. Those  
35 skilled in the art can, however, reasonably predict a suitable approach from the amino acid sequence, the presence of reactive groups, and the active site. In

WO 90/09798

PCT/US90/01031

22

applying the invention, those skilled in the art can also create genetically modified cytokines replacing amino acids with non-reactive amino acids, or vice versa to target linkage of the immobilization site. Those  
5 skilled in the art may also modify the codon of the cytokine to produce one with terminal reactive groups thereby providing a high probability of directing linkage of the immobilization site.

A functionalized surface includes reactive  
10 functional groups that provide a site for binding: (a) directly to a site on the cytokine; or (b) to a suitable linking arm. Such functional groups include hydroxyl (-OH), amino (-NH<sub>2</sub> or -NHR, wherein R is alkyl or aryl), carboxyl (-CO<sub>2</sub>H), sulfhydryl (-SH), and halogens (-F,  
15 -Cl, -Br, -I). A functionalized surface may be provided by a number of means in addition to chemical treatment of a surface. For example, blue-dyed polystyrene beads obtained from Polysciences, provide a functionalized surface despite polystyrene itself not having functional  
20 groups available for reaction. The blue dye is bound to, adsorbed on, or copolymerized with the polystyrene and provides free amino groups. A wide variety of other methods for providing suitable functional groups are known.

25 Suitable particulate supports include inorganic supports, such as, glass, quartz, ceramics, zeolites, metals, and metal oxides; polymeric materials, including homopolymers, copolymers, and oligopolymers, derived from monomeric units comprising definite units such as  
30 styrene, divinylbenzene, ethylene, butadiene, acrylonitrile, acrylic acid, methacrylic acid, esters of acrylic and methacrylic acid, vinyl acetate, fluoroalkene, acrylamide, and methacrylamide; carbohydrate supports, such as, agarose, cross-linked  
35 agarose, dextran, cross-linked dextran, inulin, hyaluronic acid, cellulose, cellulose derivatives such as carboxymethyl cellulose (CMC), starch and starch

WO 90/09798

PCT/US90/01031

23

derivatives (e.g., starch microspheres); and insoluble protein materials, such as, gelatin, collagen, or albumin.

The surface of the immobilizing support of the present invention is preferably nonporous. The use of materials having a nonporous surface, such as substantially spherical polymeric beads or microspheres, allows for binding of the cytokine to the outer surface of the support, thereby providing the cytokine in a biologically available, unhindered position. A surface is considered nonporous where the size of any pores in the material is sufficiently small so as to block or substantially hinder the migration of the cytokine into the interior of the spheres. For use as a sustained release, biodegradable formulation, a porous surface may be preferred to permit high drug loading, with new active sites exposed as the support degrades.

The size and shapes of the support may be varied widely, depending on the particular cytokine and its intended use. Polymeric spheres having a diameter of about 0.5-500  $\mu\text{m}$ , and particularly about 1-75  $\mu\text{m}$ , are preferable supports. Such supports are preferred, for example, for the in vitro growth of IL-2 dependent lymphocytes. Other preferable supports include staple fibers having a diameter of about 5-200  $\mu\text{m}$ .

#### Cytokine Linking Groups

The immobilized cytokines of the present invention preferably include a cytokine covalently bound, either directly or through a linking arm, to the support materials. It is believed that the length of the linking arm may be related to the biological activity of the bound cytokine. Suitable linking arms include one or more bifunctional linking groups such as: (1) diamines, having the formula  $\text{NH}_2\text{-R}^1\text{-NH}_2$ , where  $\text{R}^1$  is a  $\text{C}_2\text{-C}_{20}$  alkyl group; (2) amino acids, having the general formula  $\text{NH}_2\text{-R}^2\text{-CO}_2\text{H}$ , where  $\text{R}^2$  is a  $\text{C}_1\text{-C}_{20}$  alkyl

WO 90/09798

PCT/US90/01031

24

group; and (3) dialdehydes, having the formula  $\text{OHC-R}^3\text{-CHO}$ , where  $\text{R}^3$  is a  $\text{C}_1\text{-C}_{20}$  alkyl group. Two or more linking groups may be coupled to provide additional length. Examples of suitable linking groups include

5 6-aminocaproic acid, 1,6-diaminohexane, 1,12-diaminododecane, glutaraldehyde, and mixtures thereof.

In a preferred embodiment of the invention, the solid support includes a functionalized surface having a

10 plurality of reactive, exposed functional groups. The cytokine is thus directly covalently attached to a functional group on the surface, or to a linking arm of appropriate length that is covalently attached to the functional group. Following extensive washing of the

15 support having a functionalized surface, the biologically active moiety (cytokine) is attached to an exposed functional group or to the linking arm. Acceptable methods of attachment include: (1) the use of water-soluble carbodiimides in the reaction of a

20 carboxyl group on the functionalized surface of the polymeric support and a free accessible amino group on the cytokine, believed to form a stable amide bond; (2) the use of bifunctional aldehydes (e.g., glutaraldehyde) as a linking arm, which can couple an amino group on the

25 surface of the polymeric support and a free accessible amino group on the cytokine; and (3) the use of cyanogen bromide in the reaction of a hydroxyl group on the solid support with an amino group on a linking arm or on the cytokine.

30 The stability of the immobilized cytokine will depend on the nature of the covalent bond(s) between the cytokine, either directly to the immobilizing surface, or through the linking arm (if present). Stable, firmly bound cytokines will demonstrate the desired biological

35 activity through repeated uses.

WO 90/09798

PCT/US90/01031

25

The stability of the following bonds linking a protein to an insoluble matrix are considered relatively weak:

- 5  
1. Isourea (Polymer-O-C-NH-Protein)

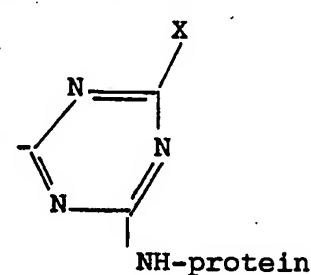
This linkage is formed from the reaction of an amino group on the protein (chiefly the lysyl side chain amine) with polyhydroxylic matrices (e.g., agarose, cellulose, and dextran) that have been activated with such reagents as cyanogen bromide (CNBr), 1-cyano-4-N,N-dimethylamine pyridinium tetrafluoroborate, and the like.

- 15  
2. Imidocarbonate (Polymer-O-C-O-Polymer)

This linkage is also formed as above for isourea, from the reaction of an amino group on the protein (chiefly the lysyl side chain) with polyhydroxylic matrices activated as above.

The stabilities of the following protein-insoluble matrix bonds are considered relatively strong:

- 30  
1. Urethane (Polymer-O-C-NH-Protein)
- This linkage is formed from the reaction of an amino group on the protein with polyhydroxylic matrices that have been activated with such reagents as 4-nitrophenyl chloroformate, N-hydroxysuccinimidyl chloroformate, carbonyl diimidazole, and the like.

- 35  
2. Triazine ether (Polymer-O-C-NH-protein)

WO 90/09798

PCT/US90/01031

26

This linkage is formed from the reaction of a protein amino group with a polyhydroxylic matrices that have been activated with such reagents as cyanuric chloride.

The stability of the following protein-insoluble matrix bonds are considered relatively very strong:

1. Amine (Polymer-NR-Protein)

This linkage is formed in a variety of ways, including the reaction of a protein amino group with (1) polyhydroxylic matrices that have been activated with such reagents as tresyl chloride, sulfonyl chloride and the like, with oxiranes (epoxides) such as bisoxirane and epichlorohydrin and with (2) polyamino matrices that have been activated with such reagents as glutaraldehyde.

2. Amide (Polymer-C(=O)-NH-Protein or Polymer-NH-C(=O)-Protein)

This linkage can be formed in a variety of ways, including the reaction of a protein amino group with an activated carboxyl group on an insoluble matrix. Activation of these carboxyl groups can be achieved via formation of "active" esters (e.g., N-hydroxysuccinimide, p-nitrophenol, or pentachlorophenol) or by reaction with carbodiimides. Conversely, an amide bond may also be formed by the reaction of an amino group on an insoluble matrix with a suitably activated (e.g., a water soluble carbodiimide) carboxyl group on the protein, especially the aspartic acid and glutamic acid side chain carboxyl groups.

It is preferred that the covalent attachment be directed to a single site on the cytokine, preferably a suitable distance from the biologically active site. This consideration may dictate the preferred choice of linking arms and the specific chemistry chosen in the attachment



WO 90/09798

PCT/US90/01031

27

of the linking arms, support, and cytokine to optimize biological activity.

#### Use of the Bound Cytokine

5           Bound cytokines of the present invention can be used to induce and regulate a variety of biological reactions, including for example: (1) in vitro growth and production of cellular blood components including stem cells, and cells in various stages of differentiation,  
10 including red cells, lymphocytes, macrophages, and/or neutrophils; (2) the in vitro growth and production of specialized effector cells, including lymphokine activated killer (LAK) cells, natural killer cells, subpopulations of lymphokine activated killer cells, tumor infiltrating  
15 lymphocytes, and/or cytotoxic T-cells; (3) the treatment of malignant disease by the in vivo intraperitoneal and/or intrapleural administration of the bound cytokines; (4) the treatment of malignant disease by the in vivo intravenous administration of the bound cytokines; (5) the  
20 treatment, preferably by intravenous administration or by in-situ placement of the bound cytokine, of refractory anemias, thrombocytopenias, and neutropenias associated with primary bone marrow failure or secondary bone marrow failure due, for example, to a lack of erythropoietin in  
25 chronic renal failure and/or renal failure in patients on renal dialysis; (6) the treatment of hard and soft tissue wounds by surface application of the bound cytokines or in-situ placement of the bound cytokines; and (7) treatment of osteoporosis by the in vivo intravenous  
30 administration of the bound cytokines. See S. Nakagawa and S. Yoshida et al., Differentiation, 29, 284 (1985), and J. Blay and K.D. Brown, J. Cell Physiol., 124, 107 (1985).

35           The present invention will be further described by reference to the following examples.

WO 90/09798

PCT/US90/01031

28

Example 1Attachment of IL-2 to Blue-Dyed Polystyrene Beads (9.64  $\mu$ m)

Recombinant IL-2 (Amgen, Thousand Oaks, CA, ala-125 analog) was immobilized on 9.64  $\mu$ m blue-dyed polystyrene beads (Polysciences, Warrington, PA) using a bifunctional aldehyde in the following manner. A 0.25 ml-aliquot of a 2.5% aqueous suspension of 9.64  $\mu$ m blue-dyed polystyrene beads was diluted with 1.0 ml of phosphate buffered saline (PBS, pH 7.40) and centrifuged for 5 minutes in a microcentrifuge. The supernatant was carefully removed and discarded. The beads were washed twice by suspension in 1.0 ml-portions of PBS followed by centrifugation. The beads were then suspended in 0.75 ml of an 8% solution of glutaraldehyde in PBS. Activation was allowed to proceed for 5 hours at room temperature with gentle end-over-end mixing. The reaction mixture was centrifuged and the supernatant was discarded. The pellet, i.e., the agglomerated beads, was washed twice with 1.0 ml-portions of PBS. The pellet was then suspended in 0.4 ml of PBS and treated with 0.1 ml of an aqueous IL-2 solution (100  $\mu$ g IL-2, activity 600,000 units). The reaction mixture was mixed overnight at room temperature, centrifuged, and the supernatant was carefully removed and saved. The pellet was resuspended in 0.5 ml of PBS, and the mixture was centrifuged. The supernatant was removed and added to the first supernatant. This combined supernatant solution (ca. 1.0 ml) was preserved at 4°C for the subsequent determination of residual IL-2 activity.

The beads were then processed in the following manner. The beads were suspended in 0.5 ml of 0.5 M ethanolamine in PBS and mixed for 30 minutes at room temperature. The mixture was centrifuged, the supernatant was discarded, and the pellet was washed once with 0.5 ml of PBS. The beads were suspended in 0.5 ml of 1% bovine serum albumin (BSA, Sigma, St. Louis, Mo) in PBS, mixed for 30 minutes at room temperature, and centrifuged. The

WO 90/09798

PCT/US90/01031

29

supernatant was discarded. The pellet was then washed twice with 0.5 ml-portions of the BSA/PBS solution and finally suspended in 0.5 ml of a storage buffer (sodium chloride (0.88%), BSA (1%), glycerol (5%), and sodium azide (0.1%) in 0.02 M sodium phosphate (pH 7.40)). The beads were stored at 4°C until used.

An assay of the supernatant solution for IL-2 activity revealed an activity of 50,400 units (8.4% of the activity of the original solution), indicating that 91.6% of the IL-2 had been bound to the beads.

#### Example 2

##### Attachment of IL-2 to Blue-Dyed Polystyrene Beads (0.93 $\mu$ m)

Recombinant IL-2 (Amgen, ala-125 analog, 100  $\mu$ g IL-2, activity 660,000 units) was immobilized on 0.93  $\mu$ m blue-dyed polystyrene beads (Polysciences) using a bifunctional aldehyde following the procedure described in Example 1. Because of the smaller bead size, however, longer centrifugation times (10 minutes) were required to effect the complete separation of the beads from the supernatant. Following the final washes, the beads were suspended in 0.5 ml of the storage buffer used in Example 1 and kept at 4°C until used. An assay of the supernatant solution for IL-2 activity revealed an activity of 18,000 units (2.7% of the activity of the original solution), indicating that 97.3% of the IL-2 had been bound to the beads.

#### Example 3

##### Attachment of IL-2 to Blue-Dyed Polystyrene Particles (421 $\mu$ m)

Recombinant IL-2 (Amgen, ala-125 analog) was immobilized on blue-dyed polystyrene particles (Polysciences, 421  $\mu$ m) using a bifunctional aldehyde in the following manner. Blue-dyed polystyrene particles (10 mg) were washed three times with 1.0 ml-portions of PBS (pH 7.40). They were then activated with glutaraldehyde

WO 90/09798

PCT/US90/01031

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and coupled to recombinant IL-2 (0.2 ml of aqueous IL-2 solution, 200  $\mu$ g IL-2, activity  $1.5 \times 10^6$  units) following the procedure described in Example 1. Following coupling and processing as described in Example 1, the beads were  
5 stored in 1.0 ml of the storage buffer used in Example 1 at 4°C. A determination of the IL-2 activity in the supernatant revealed an activity of 176,000 units (11.7% of the activity of the original solution), indicating that 88.3% of the IL-2 had been bound to the particles.

10

#### Example 4

##### Attachment of IL-2 to Blue-Dyed Polystyrene

##### Beads (9.64 $\mu$ m): Effect of IL-2 Solution Concentration

The effect of IL-2 (Amgen, ala-125 analog)  
15 concentration in the immobilization process was demonstrated in the following manner. The pellets obtained from eight 0.125 ml-aliquots of a 2.5% aqueous suspension of blue-dyed polystyrene beads (9.64  $\mu$ m) were washed with PBS and activated with glutaraldehyde as  
20 described in Example 1, except that the reactions were carried out at one-half the scale. The activated beads were then suspended in various amounts of PBS and IL-2, as designated in Table 2, and allowed to react at room temperature overnight. Following this coupling reaction,  
25 the beads were processed according to the procedure described in Example 1, suspended in 0.25 ml-portions of the storage buffer, and kept at 4°C until used. The supernatant solutions obtained from the various coupling reactions were assayed for the residual IL-2 activity  
30 present. The results appear in Table 2. The difference between the activity of each IL-2 solution used in the coupling reactions (before) and that recovered in the resultant supernatant (after) yielded the value of % IL-2 incorporated.

35

WO 90/09798

PCT/US90/01031

31

Example 5Attachment of IL-2 (Recombinant Natural Sequence)  
to Blue-Dyed Polystyrene Beads (9.64  $\mu$ m)

5           Recombinant IL-2 (Amgen, natural sequence) was  
immobilized on 9.64  $\mu$ m blue-dyed polystyrene beads  
following a procedure similar to that described in Example  
1. The pellet obtained from a 0.125 ml-aliquot of a 2.5%  
aqueous suspension of blue-dyed polystyrene beads was  
10 washed with three 0.5 ml-portions of PBS, activated with  
0.5 ml of 8% glutaraldehyde/PBS, and suspended in a  
solution of recombinant IL-2 (0.032 ml of aqueous IL-2  
solution, 32  $\mu$ g IL-2, activity 60,000 units) in 0.4 ml of  
PBS. After allowing the reaction to proceed by mixing at  
15 room temperature overnight, the reaction mixture was  
centrifuged and the supernatant was carefully removed and  
preserved. The pellet was resuspended in 0.5 ml of PBS  
and the mixture was centrifuged. The supernatant was  
removed and added to the first supernatant. The beads  
20 were processed following the procedure described in  
Example 1, suspended in 0.25 ml of the storage buffer, and  
kept at 4°C until used. A determination of the IL-2  
activity in the supernatant revealed an activity of 5,700  
units (9.5% of the activity of the original solution),  
25 indicating that 90.5% of the IL-2 had been bound to the  
beads.

Example 6Attachment of IL-2 to Polybead<sup>®</sup> Carboxylate  
Microspheres (9.67  $\mu$ m)

30           Recombinant IL-2 (Amgen, ala-125 analog) was  
immobilized on 9.67  $\mu$ m Polybead<sup>®</sup> carboxylate microspheres  
(Polysciences, carboxylate modified polystyrene) using a  
water-soluble carbodiimide in the following manner. The  
35 pellet obtained from a 0.25 ml-aliquot of a 2.5% aqueous  
suspension of Polybead<sup>®</sup> carboxylate microspheres was washed  
with three 1.0 ml-portions of PBS. The beads were

WO 90/09798

PCT/US90/01031

32

suspended in 0.4 ml of PBS, and 3.0 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl (EDCI, Pierce Chemicals, Rockford, IL) was added and dissolved. An aqueous solution of recombinant IL-2 (0.05 ml, 50 µg IL-2, activity 375,000 units) was then added. After mixing overnight at room temperature, the reaction mixture was centrifuged and the supernatant was carefully removed and preserved. The pellet was resuspended in 0.5 ml of PBS and the mixture was centrifuged. The supernatant was removed and added to the first supernatant. The beads were then processed according to the method described in Example 1, suspended in 0.25 ml of the storage buffer, and stored at 4°C until used. An assay for IL-2 activity in the supernatant revealed an activity of 570 units (0.2% of the activity of the original solution), indicating that 99.8% of the IL-2 had been bound to the beads.

#### Example 7

##### Attachment of IL-2 to Polybead<sup>®</sup> Carboxylate Microspheres

##### (9.67 µm) With a 6-Aminocaproic Acid Linking Arm

Recombinant IL-2 (Amgen, ala-125 analog) was immobilized on 9.67 µm Polybead<sup>®</sup> carboxylate microspheres with a 6-aminocaproic acid linking arm using a water-soluble carbodiimide in the following manner. The pellet obtained from a 0.25 ml-aliquot of carboxylate microspheres was washed as described in Example 6, suspended in 0.5 ml of PBS, and treated with 3.0 mg of N-hydroxysulfosuccinimide (sulfo-NHS, Pierce Chemicals, Rockford, IL) and 3.0 mg of EDCI. After vortexing to dissolve the reagents, the reaction mixture was gently mixed for 30 minutes at room temperature. The slurry was then centrifuged and the supernatant was discarded. The pellet was suspended in 0.5 ml of a 0.5 M solution of 6-aminocaproic acid in PBS. The resulting slurry was mixed for 20 hours at room temperature and centrifuged. The supernatant was discarded. The pellet was washed with three 0.5 ml-portions of PBS, resuspended in 0.35 ml of

WO 90/09798

PCT/US90/01031

33

PBS, and treated with 0.05 ml of an aqueous solution of IL-2 (50 µg IL-2, activity 375,000 units) and 2.0 mg of EDCI. After vortexing to dissolve the reagents, the reaction mixture was gently mixed at room temperature overnight. The slurry was then centrifuged, and the supernatant was carefully removed and saved. The pellet was resuspended in 0.6 ml of PBS, and the mixture was centrifuged. The supernatant was removed and added to the first supernatant. The beads were then processed as described in Example 1, suspended in 0.5 ml of the storage buffer, and stored at 4°C until used. A determination of the IL-2 activity present in the supernatant revealed an activity of 460 units (0.1% of the original solution), indicating that 99.9% of the IL-2 had been bound to the beads.

#### Example 8

##### Attachment of IL-2 to Polybead<sup>®</sup> Carboxylate

##### Microspheres (9.67 µm) With a

##### 1,6-Diaminohexane/Glutaraldehyde Linking Arm

Recombinant IL-2 (Amgen, ala-125 analog) was immobilized on 9.67 µm Polybead<sup>®</sup> carboxylate microspheres with a 1,6-diaminohexane/glutaraldehyde linking arm using a water-soluble carbodiimide in the following manner. The pellet obtained from a 0.25 ml-aliquot of carboxylate microspheres was washed with three 1.0 ml-portions of PBS (pH 7.40), suspended in 0.5 ml of a 0.5 M solution of 1,6-diaminohexane in PBS (pH 9.50) and treated with 3.0 mg of EDCI. The slurry was vortexed to dissolve the reagents and mixed for 20 hours at room temperature. This reaction mixture was centrifuged, the supernatant was discarded, and the pellet was washed with three 0.5 ml-portions of PBS (pH 7.40). The pellet was then suspended in 0.5 ml of 8% glutaraldehyde in PBS and mixed for 4 hours at room temperature. The slurry was centrifuged, the supernatant was discarded, and the pellet was washed again with three 0.5 ml-portions of PBS. The resulting pellet was then

WO 90/09798

PCT/US90/01031

34

suspended in 0.35 ml of PBS and treated with 0.05 ml of an aqueous solution of IL-2 (50  $\mu$ g IL-2, activity 375,000 units). The slurry was mixed overnight at room temperature, centrifuged, and the supernatant was  
5 carefully removed and preserved. The pellet was resuspended in 0.6 ml of PBS, and the mixture was centrifuged. The supernatant was removed and added to the first supernatant. The beads were then processed as described in Example 1, suspended in 0.5 ml of the storage  
10 buffer, and stored at 4°C until used. A determination of the IL-2 activity present in the supernatant revealed 50,000 units (13.3% of the original), indicating that 86.7% of the IL-2 had been bound to the beads.

15

Example 9Attachment of IL-2 to Polybead<sup>®</sup> CarboxylateMicrospheres (9.67  $\mu$ m) With a1,12-Diaminododecane/Glutaraldehyde Linking Arm

Recombinant IL-2 (Amgen, ala-125 analog) was  
20 immobilized on 9.67  $\mu$ m Polybead<sup>®</sup> carboxylate microspheres with a 1,12-diaminododecane/glutaraldehyde linking arm using a water-soluble carbodiimide in the following manner. The pellet obtained from 0.25 ml of carboxylate  
25 microspheres was washed with PBS (pH 7.40, 3 x 1.0 ml), suspended in 0.75 ml of 0.2 M 1,12-diaminododecane in PBS (pH 7.0), and treated with 5.0 mg of EDCI. After mixing for 18 hours at room temperature, the reaction mixture was centrifuged and the supernatant was discarded. The pellet  
30 was washed with PBS (pH 7.40, 3 x 1.0 ml), and activated with 1.0 ml of 8% glutaraldehyde in PBS as described in Example 8. After activation, the slurry was centrifuged, the supernatant was discarded, and the pellet was washed again with three 0.5 ml-portions of PBS. The resulting  
35 pellet was then suspended in 0.4 ml of PBS, treated with 0.1 ml of an aqueous IL-2 solution (100  $\mu$ g IL-2, activity 750,000 units). The mixture was allowed to react overnight at room temperature. The slurry was centrifuged



WO 90/09798

PCT/US90/01031

35

and the supernatant was carefully removed and preserved. The pellet was resuspended in 0.5 ml of PBS, and the mixture was centrifuged. The supernatant was removed and added to the first supernatant. The beads were then  
5 processed as described in Example 1, suspended in 0.5 ml of the storage buffer, and stored at 4°C until used. A determination of the IL-2 activity present in the supernatant showed an activity of 42,000 units (5.6% of the original), indicating that 94.4% of the IL-2 had been  
10 bound to the beads.

#### Example 10

##### Attachment of IL-2 to Polybead® Carboxylate

##### Microspheres (65 ± 25 µm) With a

##### 1,12-Diaminododecane/Glutaraldehyde Linking Arm

Recombinant IL-2 (Amgen, ala-125 analog) was immobilized on 65 ± 25 µm Polybead® carboxylate microspheres (Polysciences) with a 1,12-diaminododecane/glutaraldehyde linking arm using a water-soluble carbodiimide in the following manner. The pellet  
20 obtained from 0.50 ml of a 2.5% suspension of 65 ± 25 µm carboxylated polybeads was washed with PBS (pH 7.40, 3 x 1.0 ml), suspended in 1.0 ml of 0.2 M 1,12-diaminododecane in PBS (pH 6.00), and treated with 10 mg of EDCI. After  
25 mixing for 24 hours at room temperature, the reaction mixture was centrifuged and the supernatant was discarded. The pellet was washed with PBS (pH 7.40, 3 x 1.0 ml), and activated with 1.0 ml of 8% glutaraldehyde in PBS as described in Example 8. After activation, the slurry was  
30 centrifuged, the supernatant was discarded, and the pellet was washed again with three 0.5 ml-portions of PBS. The resulting pellet was then suspended in 0.75 ml of PBS, and treated with 0.25 ml of an aqueous IL-2 solution (0.1025 mg IL-2, activity 900,000 units). The mixture was allowed  
35 to react by mixing overnight at room temperature. The beads were processed as described in Example 1, suspended in 0.5 ml of the storage buffer, and stored at 4°C until

WO 90/09798

PCT/US90/01031

36

used. A determination of the IL-2 activity present in the supernatant revealed an activity of 144,450 units (16.0% of the original), indicating that 84% of the IL-2 had been bound to the beads.

5

Example 11

Attachment of IL-2 to Polybead<sup>®</sup> Carboxylate

Microspheres (9.67  $\mu$ m) With a

1,12-Diaminododecane Linking Arm

10 via Free Carboxyl Groups on the Cytokine

Recombinant IL-2 (Amgen, ala-125 analog) was immobilized on 9.67  $\mu$ m Polybead<sup>®</sup> carboxylate microspheres with a 1,12-diaminododecane linking arm via free carboxyl groups on IL-2 using a water-soluble carbodiimide in the following manner. The pellet obtained from 0.25 ml of carboxylate microspheres (9.67  $\mu$ m) was washed with PBS (pH 7.40, 3 x 1.0 ml), and reacted with 1,12-diaminododecane/EDCI as described in Example 9. After mixing for 18 hours at room temperature, the reaction mixture was centrifuged and the supernatant was discarded. The modified beads were then thoroughly washed with PBS (pH 7.40, 3 x 1.0 ml), resuspended in 0.4 ml of PBS, treated with 0.1 ml of an aqueous IL-2 solution (41  $\mu$ g IL-2, activity 360,000 units) followed by 5.0 mg of EDCI, and mixed overnight at room temperature. The reaction mixture was centrifuged and the supernatant was carefully removed and saved. The pellet was resuspended in 0.5 ml of PBS and the mixture was centrifuged. The supernatant was removed and added to the first supernatant. The beads were then suspended in 1.0 ml of 1% BSA/PBS and mixed for 30 minutes at room temperature. The mixture was centrifuged and the supernatant was discarded. The pellet was washed with the BSA/PBS solution (3 x 1.0 ml) and finally suspended in 0.5 ml of the storage buffer, and stored at 4°C until used. A determination of the IL-2 activity present in the supernatant revealed 834 units (0.2% of the original), indicating that 99.8% of the IL-2

WO 90/09798

PCT/US90/01031

37

had been bound to the beads.

### Example 12

5     Attachment of Polyethylene Glycol-Modified IL-2 to  
      Polybead<sup>®</sup> Carboxylate Microspheres (9.67  $\mu$ m) With a  
      1,12-Diaminododecane Linking Arm

Recombinant IL-2 (Amgen, ala-125 analog) was reacted with a ten-fold molar excess of methoxypolyethylene glycolyl N-succinimidyl glutarate (MW  
10 4800) [Abuchowski et al., Cancer Biochem. Biophys., 1, 175 (1984)] following the procedure described by Katre and Knauf in International Patent Application Number PCT/US86/01252 (International Publication Number WO87/00056), incorporated herein by reference. The  
15 modified IL-2 was purified by size exclusion chromatography on a Bio-Gel P-10 column using PBS (pH 7.40) as the eluting solvent. The purified column fraction used for this experiment contained 764,000 units of IL-2 activity per ml of buffer.

20     The modified IL-2 was immobilized on 9.67  $\mu$ m Polybead<sup>®</sup> carboxylate microspheres using a 1,12-diaminododecane linking arm in the following manner. The pellet obtained from 0.15 ml of carboxylate microspheres was reacted with 1,12-diaminododecane in the  
25 presence of EDCI following the procedure described in Example 9. After mixing for 18 hours at room temperature, the reaction mixture was centrifuged and the supernatant was discarded. The modified beads then were thoroughly washed with PBS (pH 7.40, 3 x 1.0 ml), resuspended in 0.3  
30 ml of PBS, treated with 0.3 ml of the modified IL-2 solution (activity 229,000 units) followed by 5.0 mg of EDCI, and allowed to mix at room temperature overnight. The slurry was centrifuged and the supernatant was carefully removed and saved. The pellet was resuspended  
35 in 0.5 ml of PBS and the mixture was centrifuged. The supernatant was removed and added to the first supernatant. The beads were then suspended in 1.0 ml of

WO 90/09798

PCT/US90/01031

38

1% BSA/PBS and mixed for 30 minutes at room temperature. The mixture was centrifuged and the supernatant was discarded. The pellet was washed with the BSA/PBS solution (3 x 1.0 ml) and finally suspended in 0.5 ml of the storage buffer, and stored at 4°C until used. A determination of the IL-2 activity present in the supernatant revealed 509 units (0.2% of the original), indicating that 99.8% of the IL-2 had been bound to the beads.

10

### Example 13

#### Attachment of IL-2 to Polybead<sup>®</sup> Amino Microspheres

Recombinant IL-2 (Amgen, ala-125 analog) was immobilized on 5.29  $\mu$ m Polybead<sup>®</sup> amino microspheres (Polysciences, amino functionalized polystyrene) using a bifunctional aldehyde in the following manner. The pellet obtained from a 0.25 ml-aliquot of Polybead amino microspheres was washed with PBS (3 x 0.5 ml), activated with 0.7 ml of 8% glutaraldehyde in PBS following the procedure described in Example 1. After washing the beads with PBS (3 x 0.5 ml), they were suspended in 0.4 ml of PBS, and treated with 0.1 ml of an aqueous IL-2 solution (100  $\mu$ g IL-2, 750,000 units). The mixture was mixed overnight at room temperature. The reaction mixture was then centrifuged and the supernatant was carefully removed and preserved. The pellet was resuspended in 0.5 ml of PBS, and the mixture was centrifuged. The supernatant was removed and added to the first supernatant. The beads were processed as described in Example 1, suspended in 0.5 ml of the storage buffer, and stored at 4°C until used. A determination of the IL-2 activity present in the supernatant revealed 44,500 units (5.9% of the original solution), indicating that 94.1% of the IL-2 had been bound to the beads.

35

WO 90/09798

PCT/US90/01031

39

Example 14Attachment of IL-2 to Sephadex® G-10 Particles  
(40-120 µm) With a 6-Aminocaproic Acid Spacer Arm

5           Recombinant IL-2 (Amgen, ala-125 analog) was  
immobilized on degradable Sephadex® G-10 resin particles  
(Pharmacia, Piscataway, NJ, cross-linked dextran  
particles, 40-120 µm) with a 6-aminocaproic acid linking  
arm in the following manner. A slurry of approximately  
10 7.5 ml of wet, packed Sephadex® G-10 resin in 7.5 ml of  
water was activated with 1.5 g of cyanogen bromide (CNBr)  
according to the published procedure; see P. Cuatrecasas,  
J. Biol. Chem., 245, 3059 (1970), incorporated herein by  
reference. After activation, the resin was rapidly  
15 filtered, washed with 100 ml of cold 0.2 M sodium borate  
buffer (pH 9.0), and added to 50 ml of 1.0 M 6-  
aminocaproic acid in 0.2 M sodium borate (pH 9.0). The  
mixture was mixed at room temperature for 20 hours. The  
resin was collected by filtration, washed with ca. 200 ml  
20 of H<sub>2</sub>O, and dried under high vacuum for 48 hours. A 10 mg-  
portion of the dried resin was swollen for 24 hours in 1.0  
ml of PBS. The suspension was then centrifuged, the  
supernatant discarded, and the resin washed with PBS (3 x  
1.0 ml). The pellet was resuspended in 0.4 ml of PBS,  
25 treated with 0.1 ml of an aqueous solution of IL-2 (100 µg  
IL-2, activity 750,000 units), followed by 3.0 mg of EDCI,  
and mixed overnight at room temperature. The resin was  
processed as described in Example 1, suspended in 0.5 ml  
of the storage buffer, and stored at 4°C until used. A  
30 determination of the IL-2 activity present in the  
supernatant 852 units (0.1% of the original), indicating  
that 99.9% of the IL-2 had been bound to the resin.

WO 90/09798

PCT/US90/01031

40

Example 15Attachment of IL-2 to Sephadex<sup>®</sup> G-10 With a  
1,6-Diaminohexane/Glutaraldehyde Linking Arm

5           Recombinant IL-2 (Amgen, ala-125 analog) was  
immobilized on degradable Sephadex<sup>®</sup> G-10 particles with a  
1,6-hexanediamine/glutaraldehyde linking arm in the  
following manner. Wet, packed Sephadex<sup>®</sup> G-10 resin (ca.  
7.5 ml) was activated with CNBr following the procedure  
10 described in Example 14. The washed activated resin was  
then added to 50 ml of 1.0 M 1,6-hexanediamine in 0.2 M  
sodium borate (pH 9.0). The slurry was mixed at room  
temperature for 20 hours. The resin was collected by  
filtration, washed with 200 ml of H<sub>2</sub>O, and dried under high  
15 vacuum for 48 hours. A 10 mg portion of the dried resin  
was swollen and washed, as described in Example 12. The  
pellet was activated with 1.0 ml of 8% glutaraldehyde in  
PBS as described in Example 8. After activation, the  
slurry was centrifuged, the supernatant was discarded, and  
20 the pellet was washed again with three 0.5 ml-portions of  
PBS. The activated resin was suspended in 0.4 ml of PBS,  
and treated with 0.1 ml of an aqueous solution of IL-2  
(100 µg IL-2, activity 750,000 units). The mixture was  
allowed to react with mixing overnight at room  
25 temperature. The slurry was then centrifuged and the  
supernatant was carefully removed and preserved. The  
pellet was resuspended in 0.5 ml of PBS and the suspension  
was centrifuged. The supernatant was removed and added to  
the first supernatant. The resin was then processed as  
30 described in Example 1, suspended in 0.5 ml of the storage  
buffer, and stored at 4°C until used. A determination of  
the IL-2 activity remaining in the supernatant revealed an  
activity of 29,800 units (4.0% of the original),  
indicating that 96.0% of the IL-2 had been bound to the  
35 resin.

WO 90/09798

PCT/US90/01031

41

Example 16Attachment of IL-4 to Blue-Dyed Polystyrene Beads (9.64  $\mu$ m)

Recombinant IL-4 (Amgen, natural sequence) was  
5 immobilized on 9.64  $\mu$ m blue-dyed polystyrene beads in the  
following manner. The pellet obtained from 0.25 ml of a  
2.5% suspension of blue-dyed beads was washed with PBS (pH  
7.40, 3 x 1.0 ml), and then activated with glutaraldehyde  
as described in Example 1. The beads were then suspended  
10 in 1.0 ml of a commercial IL-4 formulation containing 10.0  
 $\mu$ g IL-4 (activity  $2 \times 10^5$  units) and 0.025% human serum  
albumin (HSA) in PBS. The reaction mixture was mixed  
overnight at room temperature. Following the coupling  
reaction, the beads were processed as described in Example  
15 1, then suspended in 0.5 ml of the storage buffer and kept  
at 4°C until used. A determination of the IL-4 activity  
present in the supernatant obtained from the above  
coupling reaction could not be measured due to the lack of  
a quantifiable assay.

20

Example 17Attachment of IL-6 to Blue-Dyed Polystyrene Beads (9.64  $\mu$ m)

Recombinant IL-6 (Amgen, natural sequence) was  
immobilized on 9.64  $\mu$ m blue-dyed polystyrene beads in the  
25 following manner. The pellet obtained from 0.25 ml of a  
2.5% suspension of blue-dyed beads was washed with PBS (pH  
7.40, 3 x 1.0 ml), and then activated with glutaraldehyde  
as described in Example 1. The beads were then suspended  
in 1.0 ml of a commercial IL-6 formulation containing 10.0  
30  $\mu$ g IL-6 (activity  $1-2 \times 10^5$  units) and 0.025% HSA in PBS.  
The reaction mixture was mixed overnight at room  
temperature. Following the coupling reaction, the beads  
were processed as described in Example 1, then suspended  
in 0.5 ml of storage buffer and kept at 4°C until used.  
35 An assay of the supernatant solution from the above  
coupling reaction for IL-6 activity could not be  
quantified due to the lack of a suitable indicator cell

WO 90/09798

PCT/US90/01031

42

line.

#### Example 18

##### Attachment of Murine Granulocyte-Macrophage Colony

##### 5 Stimulating Factor to Blue-Dyed Polystyrene Beads (0.93 $\mu$ m)

Recombinant murine granulocyte-macrophage colony stimulating factor (rMuGMCSF, Amgen) was immobilized on 0.93  $\mu$ m blue-dyed polystyrene beads in the following manner. The pellet obtained from 0.25 ml of a 2.5%  
10 suspension of 0.93  $\mu$ m blue-dyed beads was washed with PBS (pH 7.40, 3 x 1.0 ml), and then activated with glutaraldehyde following the procedure described in Example 2. The beads were then suspended in 0.5 ml of a commercial rMuGMCSF formulation containing 5.0  $\mu$ g of the  
15 growth factor (activity  $5 \times 10^3$  units) and 0.025% BSA in PBS. The reaction mixture was mixed overnight at room temperature. Following the final washes, the beads were suspended in 0.5 ml of the storage buffer and kept at 4°C until used. An assay of the supernatant solution for  
20 rMuGMCSF could not be quantified due to the unavailability of an indicator cell line.

#### Example 19

##### Attachment of Human Granulocyte-Macrophage Colony

##### 25 Stimulating Factor to Blue-Dyed Polystyrene Beads (0.93 $\mu$ m)

Recombinant human granulocyte-macrophage colony stimulating factor (rHuGMCSF, Amgen) was immobilized on 0.93  $\mu$ m blue-dyed polystyrene beads in the following manner. The pellet obtained from 0.125 ml of a 2.5%  
30 suspension of 0.93  $\mu$ m blue-dyed beads was washed with PBS (pH 7.40, 3 x 1.0 ml), and then activated with glutaraldehyde as described in Example 2. The beads were then suspended in 0.6 ml of a commercial rHuGMCSF formulation that contained 3.0  $\mu$ g of the growth factor  
35 (activity 120,000 units) and 0.025% HSA in PBS. The reaction mixture was mixed overnight at room temperature. Following the final washes, the beads were suspended in



WO 90/09798

PCT/US90/01031

43

0.5 ml of storage buffer and kept at 4°C until used. An assay of the supernatant solution for GMCSF activity revealed 46 units (0.04% of the original), indicating that 99.96% of human GMCSF had been bound to the beads.

5

#### Example 20

##### Attachment of IL-3 to Blue-Dyed Polystyrene Beads (9.64 $\mu$ m)

Recombinant IL-3 (Amgen, natural sequence) was immobilized on 9.64  $\mu$ m blue-dyed polystyrene beads in the following manner. The pellet obtained from 0.25 ml of a 2.5% suspension of blue-dyed beads was washed with PBS (pH 7.40, 3 x 1.0 ml), and activated with glutaraldehyde following the procedure described in Example 1. The beads were then suspended on 0.4 ml of PBS, treated with 0.1 ml of a commercial IL-3 formulation containing 20  $\mu$ g IL-3 (activity  $2 \times 10^6$  units) and 0.025% HSA in PBS. The reaction mixture was mixed overnight at room temperature. Following processing, the beads were suspended in 0.5 ml of the storage buffer and kept at 4°C until used. An assay of the supernatant solution for IL-3 activity revealed 14,144 units (0.70% of the original), indicating that 99.3% of the IL-3 had been bound to the beads.

#### Example 21

##### Growth of CTLL-2 Cells Using Immobilized IL-2 (Recombinant Ala-125 Analog)

Samples of recombinant IL-2 (ala-125 analog) immobilized on 9.64  $\mu$ m blue-dyed polystyrene beads; 0.93  $\mu$ m blue-dyed polystyrene beads; 9.67  $\mu$ m carboxylate polystyrene beads; 9.67  $\mu$ m carboxylate polystyrene beads with 6-aminocaproic acid, 1,6-diaminohexane, and 1,12-diaminododecane linking arms; 65  $\mu$ m carboxylate polystyrene beads with 1,12-diaminododecane linking arm; 5.29  $\mu$ m amino polystyrene beads; and Sephadex® G-10 polydextran beads with 6-aminocaproic acid and 1,6-diaminohexane linking arms (see Examples 1, 2, 6, 7, 8, 9, 10, 13, 14, and 15) were examined to determine if

WO 90/09798

PCT/US90/01031

44

immobilized IL-2 would support in vitro growth of the IL-2 dependent cell line CTLL-2, a cytotoxic T-lymphocyte cell line.

The samples of beads comprising immobilized IL-2 were washed 3 times by suspension and centrifugation in a Beckman Microfuge in RPMI-1640 tissue culture medium (Whittaker M. A. Bioproducts, Inc., Walkersville, MD) containing 4% antibiotics (Fungi-Bact Solution, Irvine Scientific, Santa Anna, CA). The IL-2 immobilized beads were resuspended in RPMI-1640 medium and used for in vitro growth experiments. Aliquots of the beads were added to individual wells in a 96-well flat-bottomed tissue culture plate (Falcon #3075, Becton Dickinson & Co., Rutherford, NJ) followed by the addition of  $1 \times 10^4$  CTLL-2 cells (an IL-2 growth dependent cell line (TIB-214) obtained from American Type Culture Collection, Rockville, MD). The Sephadex® G-10 beads with immobilized IL-2 were very irregularly shaped and settled so fast it was impossible to accurately determine a bead/cell number. Therefore, fixed volumes of freshly vortexed beads were used in the experiment. The IL-2 immobilized beads and the CTLL-2 cells were incubated for 48 hours in a 37°C incubator with a 5% CO<sub>2</sub> atmosphere. After 48 hours, 1 µCi of [<sup>3</sup>H]-thymidine (ICN Biomedicals Inc., Irvine, CA) was added and the mixture was incubated for an additional 4 hours. The cells were collected via a Skatron cell harvester and counted in a liquid scintillation counter to determine the amount of cell growth as determined by [<sup>3</sup>H]-thymidine incorporation. The results are reported in Table 3 and demonstrate that all the above-listed immobilized IL-2 combinations support CTLL-2 cell growth.

#### Example 22

##### Growth of CTLL-2 Cells Using Immobilized IL-2

##### (Recombinant Natural Sequence)

Recombinant natural sequence IL-2 immobilized on 9.64 µm blue-dyed polystyrene beads was examined to

WO 90/09798

PCT/US90/01031

45

determine if it would support in vitro growth of the IL-2 dependent cell line CTLL-2. Recombinant natural sequence IL-2 was immobilized on 9.64  $\mu$ m beads as described in Example 5. The IL-2 immobilized beads were washed and  
5 assayed as described in Example 21. The result is reported in Table 4 and demonstrates that immobilized recombinant natural sequence IL-2 supports CTLL-2 cell growth.

10

### Example 23

#### Growth of CTLL-2 Cells Using Immobilized IL-2:

##### Carboxyl Group vs Amino Group Attachment

Recombinant IL-2 (ala-125 analog) immobilized on 9.67  $\mu$ m carboxylate beads with a 1,12-diaminododecane  
15 spacer arm attached to the IL-2 via a carboxyl group was examined to determine if it supports in vitro growth of the IL-2 dependent cell line CTLL-2. Recombinant IL-2 was immobilized on 9.67  $\mu$ m carboxylate beads with a 1,12-diaminododecane spacer via carboxyl groups on the IL-2  
20 molecule as described in Example 11. The immobilized IL-2 beads were washed and assayed as described in Example 21. The growth of CTLL-2 cells using IL-2 immobilized via a carboxyl group on the IL-2 was compared to the growth of CTLL-2 cells using IL-2 immobilized via an amino group on  
25 the IL-2 (as described in Example 1). The results are reported in Table 5 and demonstrate that IL-2 attached via a carboxyl group to a bead supports CTLL-2 growth and appears more active than IL-2 attached via an amino group to a bead (see Figure 1).

30

### Example 24

#### Growth of CTLL-2 Cells Using Immobilized

##### Polyethylene Glycol Modified IL-2

Chemically modified (polyethylene glycol)  
35 recombinant IL-2 (ala-125 analog) immobilized on 9.67  $\mu$ m carboxylate polystyrene beads with a 1,12-diaminododecane spacer group was examined to determine if it supports in

WO 90/09798

PCT/US90/01031

46

vitro growth of the IL-2 dependent cell line CTLL-2. IL-2 was chemically modified and immobilized according to the procedure outlined in Example 12. The immobilized chemically modified IL-2 beads were washed and assayed as described in Example 21. The results of the cell growth are shown in Table 6 and demonstrate that PEG-IL-2 beads support CTLL-2 growth.

#### Example 25

##### 10 Concentration Dependence of Immobilized Recombinant IL-2 on Growth of CTLL-2 Cells

The effect of the concentration (units/ml or mg/ml) of recombinant IL-2 (ala-125 analog), immobilized on polystyrene beads, on the growth of CTLL-2 cells was examined. Recombinant IL-2 was immobilized on 9.64  $\mu$ m blue-dyed polystyrene beads as described in Example 4. These beads were washed and assayed as described in Example 21. Concentrations of 1 and 10 beads per cell were used. Under these conditions, growth of the CTLL-2 cells was determined to be concentration dependent (see Figure 2).

#### Example 26

##### Growth of CTLL-2 Cells vs. Time

##### 25 Using Immobilized IL-2

The growth of CTLL-2 cells on immobilized recombinant IL-2 (ala-125 analog) was measured as a function of time and compared to the growth of CTLL-2 cells on soluble IL-2. Recombinant IL-2 was immobilized on 9.64  $\mu$ m blue-dyed polystyrene beads as described in Example 1. The beads were washed as described in Example 21. Aliquots of IL-2 immobilized beads (1, 5, and 10 beads/cell) were added to individual wells in a 96-well flat-bottomed tissue culture plate followed by the addition of  $1 \times 10^4$  CTLL-2 cells (an IL-2 growth dependent cell line). The beads containing immobilized IL-2 and the CTLL-2 cells were incubated for various times in a 37°C

WO 90/09798

PCT/US90/01031

47

incubator with a 5% CO<sub>2</sub> atmosphere. At the end of each time period, 1  $\mu$ Ci of [<sup>3</sup>H]-thymidine was added and the mixture was incubated for an additional 4 hours. The cells were collected using a Skatron cell harvester and counted in a liquid scintillation counter to determine cell growth. The results are graphically presented in Figure 3 along with the results of an analysis using soluble IL-2 (100 units/ml and 1000 units/ml). These results demonstrate that the growth of the CTLL-2 cells using immobilized IL-2 was comparable to or better than the growth of the CTLL-2 cells using the control, i.e., soluble IL-2. With one bead/cell, growth is not as dramatic in the 24 to 120 hour range as soluble IL-2, but growth remains steady up to 168 hours.

#### Example 27

##### Growth of CTLL-2 Cells on Recycled IL-2 Immobilized Beads

Recombinant IL-2 immobilized on 9.64  $\mu$ m blue-dyed polystyrene beads was prepared as described in Example 1, and washed as described in Example 21. These IL-2 immobilized beads were tested for their ability to be reused and to maintain long term cell cultures. Aliquots of IL-2 immobilized beads were added to sterile 1.5 ml screw cap microfuge tubes (Sarstedt Inc., Princeton, NJ), inoculated with 1 x 10<sup>4</sup> CTLL-2 cells, and incubated for 72 hours in a 37°C incubator with 5% CO<sub>2</sub> atmosphere. To several of the cultures, 1  $\mu$ Ci of [<sup>3</sup>H]-thymidine was added and the mixture was incubated for an additional 4 hours. The cells were collected via Skatron cell harvester and counted in a liquid scintillation counter to determine cell growth. The remaining cultures were centrifuged for 5 minutes in a Beckman microfuge and the supernatant was removed and discarded. These cultures were then washed 5 times with 1 ml of RPMI-1640 tissue culture medium containing 4% antibiotics, stirred by vigorous vortexing, and centrifuged (this procedure eliminates over 90% of the cells). After the fifth washing, the IL-2 immobilized

WO 90/09798

PCT/US90/01031

48

beads were resuspended in fresh medium, fresh CTLL-2 cells were added, and the 72 hour growth cycle was repeated. This procedure was repeated several times. The results are presented in Table 7, which demonstrates that IL-2  
5 immobilized beads supported growth of CTLL-2 cells for four 72 hour growth cycles while soluble IL-2 could only support significant CTLL-2 growth for two cycles.

#### Example 28

#### 10 Growth of Human Peripheral Blood Lymphocytes on Immobilized Recombinant IL-2

The growth of human peripheral blood lymphocytes (PBL's) on immobilized recombinant IL-2 (ala-125 analog) was examined. Recombinant IL-2 was immobilized on 9.64  $\mu\text{m}$   
15 blue-dyed polystyrene beads as described in Example 1. The immobilized IL-2 beads were prepared as described in Example 21 and used in the following experiment. PBL's were isolated from healthy donors by the following procedure. Lymphocytes were isolated from heparinized  
20 blood after centrifugation over LeucoPREP (Becton Dickinson & Co.) cell separation medium. The crude lymphocyte preparation was washed 3 times by centrifugation in RPMI-1640 tissue culture medium containing 4% antibiotics and 5% human AB serum (heat  
25 inactivated, North American Biologicals, Inc., Miami, FL).  $2 \times 10^5$  PBL's were added to various concentrations of IL-2 immobilized beads. The cells were incubated for various time periods in a 37°C incubator with a 5% CO<sub>2</sub> atmosphere. At the end of each time period, 1  $\mu\text{Ci}$  of [<sup>3</sup>H]-thymidine was  
30 added and the mixture was incubated for an additional 4 hours. The cells were collected via Skatron cell harvester and counted in a liquid scintillation counter to determine cell growth. The results are presented graphically in Figure 4. This example demonstrates that  
35 PBL's grow using immobilized IL-2, and that the growth of the PBL's is equal to or better than control soluble IL-2, especially after 72 hours of culture.

WO 90/09798

PCT/US90/01031

49

Example 29LAK Cell Activity Induced by Soluble Recombinant IL-2 or Immobilized IL-2

5 Human PBL's grown on immobilized recombinant IL-2 (ala-125 analog) were examined to determine if they exhibit lymphokine-activated killer (LAK) cell activity. Human PBL's were isolated as described in Example 28, activated for 96 hours with IL-2 immobilized beads  
10 prepared as described in Example 1, and washed as described in Example 21. The LAK cell killing activity was assayed using the cell targets K562, Raji, and Daudi. The assay for LAK cell killing used a 4 hour <sup>51</sup>Cr release assay that has been described in the literature. See T.  
15 L. Whiteside et al., Cancer Immunol. Immunother., 26, 1 (1988); H. F. Pross et al., J. Clinical Immunology, 1, 51 (1981). Normal NK (natural killer) cells isolated from fresh PBL's killed K652 cells but did not kill Raji or Daudi cells when they were in an activated state. The  
20 results are reported in Table 8. IL-2 immobilized beads activated LAK cells killed K562, Raji, and Daudi cells. Killing was equal to soluble IL-2 activated LAK cells.

Example 30NK/LAK Activity Induced by Immobilized IL-2

25 Recombinant IL-2 (ala-125 analog) immobilized on 9.64  $\mu$ m blue-dyed polystyrene beads (Example 1) and 65  $\mu$ m polystyrene beads (Example 10) were examined to determine if they stimulate murine lymphocytes in an ex vivo  
30 experiment to increase natural killing (NK) or lymphokine-activated killing (LAK) of a target cell line. That is, an ex vivo experiment was conducted to determine if the immobilized IL-2 beads could activate the host's immune system in the same manner that soluble IL-2 can activate  
35 LAK cell production in vivo. The experiment was performed as follows: Mature Balb/C male mice (groups of three, 17 weeks old) were injected i.p. with 200  $\mu$ l of PBS, 50,000

WO 90/09798

PCT/US90/01031

50

units recombinant soluble IL-2, 200,000 units IL-2 immobilized on 9.64  $\mu$ m blue beads (Example 1), or 100,000 units IL-2 immobilized on 65  $\mu$ m beads (Example 10). After 96 hours, cells from the peritoneal cavity and spleens  
5 were collected and assayed for NK/LAK cell activity. Splenocytes were prepared from fresh spleens as described by M. H. Zaroukian et al., Immunol. Invest., 15, 813 (1986) and C. W. Gilbert et al., J. Immunol., 140, 2821 (1988). NK/LAK cell activity was assayed by a 4 hour  $^{51}\text{Cr}$   
10 release assay, also described in the above references. The results of the ex vivo experiment are summarized in Table 9. This data indicates that soluble IL-2 activates murine splenocytes as expected, and immobilized IL-2 on 65  $\mu$ m beads also activate LAK cells in the peritoneal cavity.  
15 The LAK cell activity in the peritoneal cavity appears to be localized and may have a therapeutic value in the localized treatment of cancer.

#### Example 31

##### 20      Growth of Human Peripheral Blood Lymphocytes          on Immobilized Recombinant IL-4

Recombinant IL-4 was immobilized on 9.64  $\mu$ m blue-dyed polystyrene beads as described in Example 16. The immobilized IL-4 beads were washed as described in  
25 Example 21, and used in a PHA (phytohaemagglutinin) costimulation experiment to induce T-cell proliferation. Peripheral blood lymphocytes were obtained from healthy donors. An enriched T-cell population was isolated from lymphocytes that were isolated from heparinized blood and  
30 separated over a Ficoll gradient (LSM, Lymphocyte Separation Medium, Organon Teknika Corp., Durham, NC). Crude lymphocytes were incubated in plastic tissue culture flasks at 37°C in RPMI-1640 medium containing 5% heat inactivated human AB serum for 1 hour to remove monocytes  
35 and other adherent cells that interfere with the costimulation T-cell proliferation assay. This step was repeated twice. Nonadherent lymphocytes, enriched with T-



WO 90/09798

PCT/US90/01031

51

cells were then used in a PHA co-stimulation proliferation assay.  $1 \times 10^5$  cells were added to each well, in addition to soluble IL-4 (100 units/ml), PHA (0.05  $\mu$ g/ml), PHA plus soluble IL-4 (100 units/ml and 1 unit/ml), or PHA plus immobilized IL-4 on beads (0.5 and 1 bead/cell starting concentration), and incubated for 96 hours at 37°C. After 96 hours, the cultures were pulsed with [ $^3$ H]-thymidine for 4 hours to determine T-cell proliferation. The results are listed in Table 10 and indicate that immobilized IL-4 beads stimulate T-cell proliferation over background suboptimal PHA levels.

### Example 32

#### Growth of Human Peripheral Blood Lymphocytes on Immobilized Recombinant IL-6

Recombinant IL-6 was immobilized on 9.64  $\mu$ m blue-dyed polystyrene beads as described in Example 17. The immobilized IL-6 beads were washed as described in Example 21, and used in a PHA (phytohaemagglutinin) co-stimulation experiment to induce T-cell proliferation. Peripheral blood lymphocytes were obtained from healthy donors. An enriched T-cell population was isolated from lymphocytes that were isolated from heparinized blood and separated over a Ficoll gradient (LSM, Lymphocyte Separation Medium). Crude lymphocytes were incubated in plastic tissue culture flasks at 37°C in RPMI-1640 containing 5% heat inactivated human AB serum for 1 hour to remove monocytes and other adherent cells that interfere with the costimulation T-cell proliferation assay. This step was repeated twice. Nonadherent lymphocytes, enriched with T-cells were then used in a PHA co-stimulation proliferation assay.  $1 \times 10^5$  cells were added to each well, in addition to nothing, soluble IL-6 (100 units/ml), PHA (0.05  $\mu$ g/ml), PHA plus soluble IL-6 (100 units/ml and 1 unit/ml), or PHA plus immobilized IL-6 on beads (0.5 and 1 bead/cell starting concentration), and incubated for 96 hours at 37°C. After 96 hours, the

WO 90/09798

PCT/US90/01031

52

cultures were pulsed with [<sup>3</sup>H]-thymidine for 4 hours to determine T-cell proliferation. The results are tabulated in Table 11 and indicate that immobilized IL-6 beads stimulate T-cell proliferation over background suboptimal PHA levels.

### Example 33

#### Growth of AML-193 Cells on Immobilized

##### Recombinant Human GMCSF

Recombinant human GMCSF (rHuGMCSF) immobilized on 0.93  $\mu$ m blue-dyed polystyrene beads was examined to determine if it would support in vitro growth of a GMCSF dependent cell line AML-193. Recombinant human GMCSF was immobilized on 0.93  $\mu$ m blue-dyed beads as described in Example 19. The immobilized recombinant human GMCSF beads were washed as described in Example 21. The growth assay for AML-193 cell line was as follows. Aliquots of the washed beads were added to individual wells in a 96-well flat-bottomed tissue culture plate followed by the addition of  $1 \times 10^4$  AML-193 cells (an IL-3/GMCSF dependent cell line obtained from American Type Culture Collection, Rockville, MD). The beads with immobilized rHuGMCSF were incubated with the AML-193 cells for 116 hours in a 37°C incubator with 5% CO<sub>2</sub> atmosphere. After 116 hours, 1  $\mu$ Ci of [<sup>3</sup>H]-thymidine was added and the mixture was incubated for an additional 4 hours. The cells were collected as described in Example 21. The results are reported in Table 12 and demonstrate that immobilized recombinant human GMCSF supports AML-193 cell growth.

30

### Example 34

#### Growth of AML-193 Cells on Immobilized Recombinant IL-3

Recombinant IL-3 immobilized on 9.64  $\mu$ m blue-dyed polystyrene beads was examined to determine if it would support in vitro growth of an IL-3/GMCSF dependent cell line AML-193. Recombinant IL-3 was immobilized on 9.64  $\mu$ m blue-dyed beads as described in Example 20. The

35

WO 90/09798

PCT/US90/01031

53

immobilized IL-3 beads were washed as described in Example 21 and assayed as described in Example 33. The results are reported in Table 13 and demonstrate that immobilized recombinant IL-3 supports AML-193 cell growth.

5

#### Example 35

##### Attachment of IL-1-beta to Blue-Dyed

##### Polystyrene Beads (9.64 $\mu$ m)

Recombinant IL-1-beta (Amgen) was immobilized on  
10 9.64  $\mu$ m blue-dyed polystyrene beads in the following manner. The pellet obtained from 0.15 ml of a 2.5% suspension of blue-dyed beads was washed with PBS (pH 7.40, 3 x 1.0 ml), then activated with glutaraldehyde as described in Example 1. The beads were then suspended in  
15 0.46 ml of PBS, treated with 0.04 ml of a commercial IL-1-beta formulation containing 8.0  $\mu$ g IL-1-beta (activity 4 x 10<sup>6</sup> units) and 0.025% HSA in PBS. The reaction mixture was mixed for 24 hours at room temperature. Following the coupling reaction, the beads were processed as described  
20 in Example 1, then suspended in 0.5 ml of the storage buffer and kept at 4°C until used.

#### Example 36

##### Attachment of IL-1-alpha to Blue-Dyed

##### Polystyrene Beads (9.64 $\mu$ m)

Human sequence IL-1-alpha (R & D Systems) was immobilized on 9.64  $\mu$ m blue-dyed polystyrene beads in the following manner. The pellet obtained from 0.20 ml of a 2.5% suspension of blue-dyed beads was washed with  
30 phosphate buffered saline (PBS) (pH 7.40, 3 x 1.0 ml), and then activated with glutaraldehyde as described in Example 1. The activated beads were suspended in 0.42 ml of PBS, then treated with 0.08 ml of a formulation that contained 8.0  $\mu$ g of the cytokine and 200  $\mu$ g human serum albumin  
35 (HSA) in PBS. The reaction mixture was mixed for 24 hours at room temperature. Following the coupling reaction, the beads were centrifuged, washed with PBS (0.5 ml), then

WO 90/09798

PCT/US90/01031

54

treated with ethanolamine as described in Example 1. The beads were then washed (3 x 1.0 ml) with a solution containing 0.1% sodium dodecyl sulfate (SDS) in PBS in an effort to remove the last traces of any noncovalently bound cytokine. Following these washes, the beads were further processed as described in Example 1, then suspended in 0.5 ml of the storage buffer and kept at 4°C until used.

10

Example 37

Attachment of Recombinant Human Granulocyte  
Colony Stimulating Factor (rHuGCSF) to Blue-Dyed  
Polystyrene Beads (9.64  $\mu$ m)

Recombinant human GCSF (rHuGCSF, Amgen) was immobilized on 9.64  $\mu$ m blue-dyed polystyrene beads in the following manner. The pellet obtained from 0.20 ml of a 2.5% suspension of blue-dyed beads was washed with PBS (pH 7.40, 3 x 1.0 ml), then activated with glutaraldehyde as described in Example 1. The activated beads were suspended in 0.3 ml PBS and treated with 0.2 ml of a commercial rHuGCSF formulation containing 0.5  $\mu$ g (activity  $1 \times 10^5$  units) of the growth factor and 0.025% HSA in 0.01 M sodium acetate (pH 5.4). The suspension was mixed overnight at room temperature. Following the coupling reaction, the beads were processed as described in Example 1, then suspended in 0.5 ml of the storage buffer and kept at 4°C until used.

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Example 38

Attachment of Recombinant Murine Granulocyte  
Macrophage Colony Stimulating Factor (rMuGMCSF) to  
Blue-Dyed Polystyrene Beads (0.93  $\mu$ m)

Recombinant murine GMCSF (Amgen) was immobilized on 0.93  $\mu$ m blue-dyed polystyrene beads in the following manner. The pellet obtained from 0.25 ml of a 2.5% suspension of blue-dyed beads was washed with PBS (pH 7.40, 3 x 1.0 ml), then activated with 8% glutaraldehyde

WO 90/09798

PCT/US90/01031

55

as described in Examples 1 and 2. The activated beads were then suspended in 0.50 ml of a commercial rMuGMCSF formulation containing 5.0  $\mu\text{g}$  (activity  $5 \times 10^3$  units) of the growth factor and 0.025% BSA in PBS. The suspension  
5 was mixed for 24 hours at room temperature. Following the coupling reaction, the beads were processed as described in Examples 1 and 2, then suspended in 0.5 ml of the storage buffer and kept at 4°C until used.

10

Example 39Covalent Attachment/Adsorption of rMuGMCSF  
to Blue-Dyed Polystyrene Beads (0.93  $\mu\text{m}$ )

The pellets obtained from two 0.2 ml-portions of a 2.5% suspension of 0.93  $\mu\text{m}$  blue-dyed beads were washed  
15 with PBS (3 x 1.0 ml). One pellet (labeled C) was then activated with 1.0 ml of 8.0% glutaraldehyde in PBS for 20 hours at room temperature as described in Examples 1 and 2. The other pellet (labeled A) was suspended in 1.0 ml of PBS and also mixed for 20 hours. Both suspensions were  
20 centrifuged and the pellets washed with PBS (3 x 1.0 ml). Each pellet was then suspended in a 0.1 ml-portion of PBS and treated with 0.4 ml-portions (4.0  $\mu\text{g}$ , activity 4000 units) of the commercial rMuGMCSF formulation used in Example 38. The suspensions were then mixed overnight at  
25 room temperature, centrifuged, and the supernatants removed and saved. The two pellets were again suspended in 0.5 ml- portions of PBS, centrifuged, and the supernatants removed and added to the first supernatants (labeled A1 and C1, both ca. 1.0 ml). The pellets were  
30 then treated with 1.0 ml- portions of 0.5 M ethanolamine as described in Example 1. The supernatants (labeled A2 and C2) were removed and saved. The pellets were then suspended in 1.0 ml-portions of PBS, centrifuged, and the supernatants (labeled A3 and C3) were removed and saved.  
35 The pellets were then suspended three times in 1.0 ml- portions of 0.1% SDS/PBS, mixed for one hour, centrifuged, and the supernatants (labeled A4, A5, A6, C4, C5, and C6,

WO 90/09798

PCT/US90/01031

56

respectively) were removed and saved. The pellets were washed with 1.0 ml-portions of PBS, and the supernatants (labeled A7 and C7) were removed and saved. The pellets were then treated with 1% BSA/PBS as described in Example 1, and the various supernatants (labeled A8, A9, A10, C8, C9, and C10, respectively) were removed and saved. The beads were finally suspended in 0.5 ml of the storage buffer and, together with the supernatants, kept at 4°C until used.

10

#### Example 40

##### Attachment of Recombinant Human Insulin-like Growth Factor I (rHuILGF-I) to Blue-Dyed Polystyrene Beads (9.64 $\mu$ m)

Recombinant human insulin-like growth factor I (rHuILGF-I, Somatomedin C, available from Bachem) was immobilized on 9.64  $\mu$ m blue-dyed polystyrene beads in the following manner. The pellet obtained from 0.2 ml of a 2.5% suspension of blue-dyed beads was washed with PBS (pH 7.40, 3 x 1.0 ml) and then activated with 1.0 ml of 8.0% glutaraldehyde in PBS as described in Example 1. The washed, activated beads were suspended in 0.42 ml PBS and treated with 0.08 ml of a solution that contained 20.0  $\mu$ g of the commercial growth factor in sterile water. The suspension was mixed for 20 hours at room temperature. Following the coupling reaction, the beads were processed as described in Example 1, then suspended in 0.5 ml of the storage buffer and kept at 4°C until used.

30

#### Example 41

##### Attachment of Recombinant Human Insulin-like Growth Factor II (rHuILGF-II) to Blue-Dyed Polystyrene Beads (9.64 $\mu$ m)

Recombinant human insulin-like growth factor II (rHuILGF-II, available from Bachem) was immobilized on 9.64  $\mu$ m blue-dyed polystyrene beads in the following manner. The pellet obtained from 0.2 ml of a 2.5% suspension of blue-dyed beads was washed with PBS (3 x 1.0

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WO 90/09798

PCT/US90/01031

57

ml) and then activated with 1.0 ml of 8.0% glutaraldehyde/PBS as described in Example 1. The washed, activated beads were then suspended in 0.45 ml PBS and treated with 0.05 ml of a solution that contained 12.5  $\mu$ g of the growth factor in sterile water. The suspension was mixed for 24 hours at room temperature. Following the coupling reaction, the beads were processed as described in Example 1, then suspended in 0.5 ml of the storage buffer and kept at 4°C until used.

10

#### Example 42

#### Attachment of Recombinant Human Tumor Necrosis Factor (TNF-alpha/Cachectin) to Blue-Dyed Polystyrene

##### Beads (9.64 $\mu$ m)

Recombinant human TNF-alpha (Amgen) was immobilized on 9.64  $\mu$ m blue-dyed beads in the following manner. The pellet obtained from 0.2 ml of a 2.5% suspension of blue-dyed beads was washed with PBS (3 x 1.0 ml), then activated with glutaraldehyde as described in Example 1. Following the activation, the washed beads were suspended in 0.46 ml PBS and treated with 0.04 ml of a commercial recombinant human TNF-alpha formulation containing 19.2  $\mu$ g (activity  $1.92 \times 10^5$  units) of the growth factor in a 0.04 M Tris/0.1 M NaCl buffer (pH 8.60). The suspension was mixed for 24 hours at room temperature. Following the coupling reaction, the beads were processed as described in Example 1, then suspended in 0.5 ml of the storage buffer and kept at 4°C until used.

30

#### Example 43

#### Attachment of Fibroblast Growth Factor Basic (FGFb) to Blue-Dyed Polystyrene Beads (2.85 $\mu$ m)

Fibroblast Growth Factor Basic (Amgen) was immobilized on 2.85  $\mu$ m blue-dyed beads in the following manner. The pellet obtained from 0.2 ml of a 2.5% suspension of blue-dyed beads was washed with PBS (3 x 1.0 ml), then activated with glutaraldehyde as described in

35

WO 90/09798

PCT/US90/01031

58

Example 1. Following activation, the washed beads were suspended in 0.44 ml PBS, then treated with 0.06 ml of a commercial FGFb formulation containing 30 µg of the growth factor in a 0.02 M sodium citrate/0.1 M sodium chloride buffer (pH 5.0). The suspension was mixed for 24 hours at room temperature. Following the coupling reaction, the beads were processed as described in Example 39, then suspended in 0.5 ml of the storage buffer, and together with the various supernatants, kept at 4°C until used.

10

#### Example 44

##### Attachment of Transforming Growth Factor-beta-2 (TGF-beta-2) to Blue-Dyed Polystyrene Beads (2.85 µm)

TGF-beta-2 (R & D Systems) was immobilized on 2.85 µm blue-dyed beads in the following manner. The pellet obtained from 0.2 ml of a 2.5% suspension of blue-dyed beads was washed with PBS (3 x 1.0 ml), then treated with glutaraldehyde as described in Example 1. Following activation, the washed beads were suspended in 0.35 ml PBS, treated with 0.15 ml of a solution containing 7.5 µg of the growth factor in 0.01% Triton X-100. The suspension was mixed for 18 hours at room temperature. Following the coupling reaction, the beads were processed as described in Example 39, then suspended in 0.5 ml of the storage buffer and, together with the various supernatants, kept at 4°C until used.

#### Example 45

##### Attachment of Recombinant Human Interferon-alpha-2A (Roferon® A) to Blue-Dyed Polystyrene Beads (2.85 µm)

Recombinant human Interferon-alpha-2A (Roferon® A, Roche Laboratories) was immobilized on 2.85 µm blue-dyed polystyrene beads in the following manner. The pellet obtained from 0.2 ml of 2.5% suspension of blue-dyed beads was washed with PBS (3 x 1.0 ml), then treated with glutaraldehyde as described in Example 1. The washed, activated beads were then suspended in 0.4 ml



WO 90/09798

PCT/US90/01031

59

PBS and treated with 0.1 ml (activity  $6 \times 10^5$  units) of a commercial recombinant human Interferon-alpha-2A aqueous formulation containing 0.9 mg sodium chloride, 0.5 mg HSA, and 0.3 mg phenol. The suspension was mixed for 24 hours at room temperature. Following the coupling reaction, the beads were processed as described in Example 39, then suspended in 0.5 ml of the storage buffer and, together with the various supernatants, kept at 4°C until used.

10

#### Example 46

##### Attachment of Recombinant Human Epidermal Growth Factor (rHuEGF) to Blue-Dyed Polystyrene Beads (0.93 $\mu$ m)

Recombinant human EGF (rHuEGF, available from Amgen) was immobilized on 0.93  $\mu$ m blue-dyed polystyrene beads in the following manner. The pellet obtained from 0.2 ml of a 2.5% slurry of blue-dyed beads was washed with PBS (3 x 1.0 ml), then treated with glutaraldehyde as described in Examples 1 and 2. The washed, activated beads were suspended in 0.35 ml PBS, then treated with 0.15 ml of a solution that contained 25.0  $\mu$ g of the growth factor in PBS (pH 7.40). The suspension was mixed for 18 hours at room temperature. Following the coupling reaction, the beads were processed as described in Examples 1 and 2, then suspended in 0.5 ml of the storage buffer and kept at 4°C until used.

#### Example 47

##### Attachment of Recombinant Human Platelet-Derived Growth Factor (rHuPDGF) to Blue-Dyed Polystyrene Beads (2.85 $\mu$ m)

Recombinant human PDGF (rHuPDGF, available from Bachem) was immobilized on 2.85  $\mu$ m blue-dyed polystyrene beads in the following manner. The pellet obtained from 0.2 ml of a 2.5% suspension of blue-dyed beads was washed with PBS (3 x 1.0 ml), then treated with glutaraldehyde as described in Example 1. Following activation, the washed beads were suspended in 0.35 ml PBS, and treated with 0.15 ml of a solution containing 15.0  $\mu$ g of the growth factor

WO 90/09798

PCT/US90/01031

60

in sterile water. The suspension was mixed for 20 hours at room temperature. Following the coupling reaction, the beads were processed as described in Example 1, then suspended in 0.5 ml of the storage buffer and kept at 4°C until used.

#### Example 48

##### Attachment of Recombinant Human Erythropoietin (rHuEPO) to Co-Bind™ Well Strips

Recombinant human erythropoietin (rHuEPO) was obtained from Amgen as a liquid formulation containing 5000 units activity per ml of a solution comprised of 50% glycerol in 0.025 M HEPES buffer (pH 7.20). The Co-Bind™ well strips, strips whose surfaces have been chemically modified (i.e., activated) to covalently bind proteins, were obtained from Micro Membranes, Inc., Newark, NJ.

Four wells of the 8-well strip were then filled as shown below:

Well	<u>rHuEPO</u>		<u>Buffer, mls</u>
	<u>Units</u>	<u>mls</u>	<u>50% glycerol in 0.025 M HEPES, pH 7.20</u>
A	200	0.04	0.16
B	100	0.02	0.18
C	50	0.01	0.19
D	0	0.00	0.20

The strip was covered and incubated at 35°C for 3 hours. The supernatants A-D were then removed and saved for residual activity assays. The wells were washed with buffer (2 x 0.1 ml), then treated with 0.2 ml portions of freshly prepared 1% BSA/PBS and again incubated at 35°C for one hour. These supernatants were discarded. The wells were then thoroughly washed (3 x 0.2 ml) with RPMI-7640 tissue culture medium containing 1% Fungizone, then filled with the same media. The strip was covered and kept at 4°C until used.

WO 90/09798

PCT/US90/01031

61

Example 49Growth of CTLL-2 Cells Using IL-2 Produced FromLBRM.TG6 Cells Stimulated With ImmobilizedRecombinant IL-1-beta Polystyrene Beads

5           Recombinant IL-1-beta immobilized on 9.63  $\mu$ m  
blue-dyed polystyrene beads induces the murine lymphoma  
cell line LBRM.TG6 (American Type Culture Collection Co.,  
Rockville, MD) to synthesize IL-2 which was then assayed  
in the IL-2 dependent CTLL-2 cell line. The immobilized  
10 IL-1-beta beads were washed three times by suspension and  
centrifugation as described in Example 21. IL-1-beta  
beads in conjunction with a suboptimal concentration of  
PHA [Phytoheamagglutinin P, Wellcome Foundation, Danford,  
England] (10  $\mu$ g/ml) were added to  $5 \times 10^4$  LBRM.TG6 cells  
15 [J. W. Larrick et al., J. Immunol. Methods, 79, 39 (1985)]  
in 100  $\mu$ l of Iscove's MEM, (Whittaker M.A. Bioproducts,  
Walkersville, MD) and incubated for 48 hours at 37°C in 5%  
CO<sub>2</sub>. The reaction was stopped by placing the LBRM.TG6  
cells at 4°C for 24 hours. Next, a 50  $\mu$ l-portion of the  
20 LBRM.TG6 cell supernatant was removed and added to 50  $\mu$ l  
of fresh CTLL-2 cells. The released soluble IL-2 was  
assayed to determine if it would support CTLL-2 cell  
growth. CTLL-2 cell growth was dependent on IL-2  
concentration and was measured by the up-take, and  
25 oxidation of the tetrazolium salt MTT (3-(4,5-  
dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide)  
[T. Mosmann, J. Immunol Meth., 65, 55 (1983); and M.B.  
Hansen, S.E. Nielson and K. Berg, J. Immunol Meth., 119:  
203-210(1989)] The results are reported in Table 14 and  
30 indicate that IL-1-beta beads activate the release of  
soluble IL-2 from LBRM.TG6 cells, and that the IL-2  
released by the LBRM.TG6 cells supports growth of IL-2  
dependent CTLL-2 cells.

WO 90/09798

PCT/US90/01031

62

Example 50Recombinant IL-1-alpha Immobilized on Polystyrene Beads  
Induces LBRM.TG6 Cells to Produce IL-2

5           Recombinant IL-1-alpha immobilized on 9.64  $\mu$ m  
blue-dyed polystyrene beads induces the murine lymphoma  
cell line LBRM.TG6, American Type Culture Collection, to  
synthesize IL-2 which was then assayed in the IL-2  
dependent CTLL-2 cell line. Human sequence IL-1-alpha was  
10 immobilized on 9.64  $\mu$ m blue-dyed polystyrene beads as  
described in Example 36. The immobilized IL-1-alpha beads  
were washed three times as described in Example 21. IL-  
1-alpha beads in conjunction with a suboptimal  
concentration of PHA [Phytohemagglutinin P, Wellcome  
15 Foundation, Danford, England] (10  $\mu$ g/ml) were added to 5  
 $\times 10^4$  LBRM.TG6 cells in 100  $\mu$ l of Iscove's MEM and  
incubated for 48 hours at 37°C in 5% CO<sub>2</sub>. The reaction  
was stopped by placing the LBRM.TG6 cells at 4°C for 24  
hours. Next, 50  $\mu$ l of the LBRM.TG6 cell supernatant was  
20 removed and added to 50  $\mu$ l of fresh CTLL-2 cells. The  
released soluble IL-2 was assayed as described in Example  
49. The results are reported in Table 15 and indicate  
that IL-1-alpha beads activate the release of soluble IL-  
2 from LBRM.TG6 cells, and that the IL-2 released by the  
25 LBRM.TG6 cells supports growth of IL-2 dependent CTLL-2  
cells.

Example 51Growth of AML-193 Cells on ImmobilizedRecombinant Human GCSF

30           Recombinant human GCSF (rHuGCSF) immobilized on  
9.64  $\mu$ m blue-dyed polystyrene beads was examined to  
determine if it would support in vitro growth of a growth  
factor (GCSF) dependent cell line AML-193, American Type  
35 Culture Collection. Recombinant human GCSF was  
immobilized on 9.64  $\mu$ m blue-dyed polystyrene beads as  
described in Example 37. The immobilized rHuGCSF beads

WO 90/09798

PCT/US90/01031

63

were washed as described in Example 21. The growth assay for AML-193 cell line was as follows. Aliquots of the washed beads were added to individual wells in a 96-well flat-bottomed tissue culture plate followed by the addition of  $1 \times 10^4$  AML-193 cells as in Example 33. The beads with the immobilized rHuGCSF were incubated with the AML-193 cells for 116 hours in a 37°C incubator with a 5% CO<sub>2</sub> atmosphere. [<sup>3</sup>H]-thymidine (1 µCi) was then added to each well and the mixture was incubated for an additional 4 hours. The cells were collected as described in Example 21. The results are reported in Table 16 and demonstrate that immobilized recombinant human GCSF supports AML-193 cell growth.

15

Example 52Recombinant Murine GMCSF (rMuGMCSF) Immobilized on 0.93 µm Polystyrene Beads Stimulates Granulopoiesis in BDF1 Mice

Recombinant murine GMCSF (rMuGMCSF) immobilized on 0.93 µm blue-dyed polystyrene beads stimulates granulopoiesis in BDF1 mice. Recombinant murine GMCSF was immobilized on 0.93 µm blue-dyed polystyrene beads as described in Example 38. Immobilized rMuGMCSF beads, like soluble rMuGMCSF, stimulates granulopoiesis in the peripheral blood of mice after an injection. Ishida et al., Acta. Haemat., 8, 1 (1988) recently reported that GMCSF stimulates granulopoiesis in the peripheral blood of mice after a single injection of soluble GMCSF. These experiments were repeated using immobilized rMuGMCSF to determine if immobilized rMuGMCSF is active in vivo. BDF1 mice were injected with either soluble rMuGMCSF (20 units i.p.) or immobilized rMuGMCSF (50 units i.v.). Peripheral blood was drawn from the retro-orbital sinus of BDF1 mice at 0, 6, 12, 24, 48, 72, and 96 hours and the number of neutrophils (PMN)/ml was determined from a complete blood count. The results are shown in Figure 5 and indicate that immobilized rMuGMCSF is active in vivo. Furthermore, the results indicate that the beads stimulate PMN

WO 90/09798

PCT/US90/01031

64

production in numbers (about a 2-fold increase) and rates (maximum in 12 hours with decline to initial levels within 24 to 48 hours) comparable to soluble rMuGMCSF.

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Example 53

Recombinant Murine GMCSF (rMuGMCSF) Immobilized on  
0.93  $\mu$ m Polystyrene Beads Stimulates Granulopoiesis in  
Cyclophosphamide-Treated BDF1 Mice

Recombinant murine GMCSF (rMuGMCSF) immobilized  
10 on 0.93  $\mu$ m blue-dyed polystyrene beads stimulates granulopoiesis in cyclophosphamide-treated mice. Recombinant murine GMCSF was immobilized on 0.93  $\mu$ m blue-dyed polystyrene beads as described in Example 38. Immobilized rMuGMCSF beads like soluble rMuGMCSF  
15 stimulates granulopoiesis in the peripheral blood of mice after an injection. Ishida et al., Acta. Haemat., 8, 1 (1988) recently reported that GMCSF stimulates granulopoiesis in the peripheral blood of mice after their lymphocyte population was depleted by a single injection  
20 of cyclophosphamide. Repeated doses of GMCSF help these mice to recover lymphocyte numbers 2 to 3 days faster than untreated mice. Since immobilized rMuGMCSF shows in vivo activity (Example 52), cyclophosphamide-treated mice were given either soluble rMuGMCSF or immobilized rMuGMCSF to  
25 determine the effects rMuGMCSF on neutrophil counts. The experimental protocol was as follows. BDF1 mice were injected with cyclophosphamide (250 mg/Kg weight) at zero time to deplete the neutrophil cell count. Twenty-four hours later, either soluble rMuGMCSF (2 units injected  
30 i.p. every 12 hours for 6 days; or 2 units injected i.v. on days 1, 3, and 5), or immobilized rMuGMCSF (2 units injected i.v. on days 1, 3, and 5) was administered. Peripheral blood was drawn from the retro-orbital sinus of BDF1 mice at day 0, 3, 5, 7, and 9 and the number of  
35 neutrophils (PMN)/ml was determined from a complete blood count. The results are shown in Figure 6 and indicate that immobilized rMuGMCSF is active in vivo. Furthermore,

WO 90/09798

PCT/US90/01031

65

rMuGMCSF beads stimulate PMN production in numbers and rates comparable to soluble rMuGMCSF.

#### Example 54

#### 5     Covalently Linked rMuGMCSF Polystyrene Beads Retain           Cytokine Activity While Adsorbed rMuGMCSF           Polystyrene Beads Do Not Retain Cytokine Activity

Recombinant murine GMCSF (rMuGMCSF) covalently attached to 0.93  $\mu$ m blue-dyed polystyrene beads retain  
10 biological activity (i.e., promote the growth of DA1-E5 cells) while rMuGMCSF adsorbed to 0.93  $\mu$ m blue-dyed polystyrene beads do not retain biological activity (i.e., DA1-E5 cells do not grow). Covalent and adsorbed rMuGMCSF blue-dyed polystyrene beads were prepared and washed as  
15 described in Example 39. Beads were washed three times before the assays described in Example 21 were performed. DA1-E5 cells an IL-3/GMCSF/EPO dependent cell line, obtained from Dr. Larry Gilbert, University of Alta, Edmonton, Alberta, Canada, were used to assay both soluble  
20 fractions of rMuGMCSF and immobilized rMuGMCSF bead fractions (covalently bound or adsorbed). The rMuGMCSF assay is as follows. DA1-E5 cells ( $1 \times 10^4$ ) were incubated with either soluble rMuGMCSF or immobilized rMuGMCSF (covalent or adsorbed) for 48 hours as described in  
25 Example 21. Either MTT or 1  $\mu$ Ci of [ $^3$ H]-thymidine was added. The mixture was incubated for an additional 4 hours. Cells were harvested as described in Example 21. When the polystyrene beads were washed with sodium dodecyl sulfate (SDS), the adsorbed rMuGMCSF was removed (Figure  
30 7). These beads no longer retained any biological activity. Covalently linked rMuGMCSF, however, did not wash off with SDS. These beads retained biological activity. The results are listed in Table 17.

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WO 90/09798

PCT/US90/01031

66

Example 55Recombinant Human Insulin-Like Growth Factor-I  
(rHuILGF-I) Immobilized on 9.63  $\mu$ m Blue-dyed  
Polystyrene Beads Stimulates a Crude Lymphocytic  
Preparation to Proliferate in Serum-Free Medium

5           Recombinant human ILGF-I was immobilized on 9.63  
           $\mu$ m blue-dyed polystyrene beads as described in Example  
          40. Immobilized rHuILGF-I beads were washed as described  
          in Example 21. Schimpff et al., Acta Endocrinologica,  
10    102, 21 (1983) disclose that ILGF-I in conjunction with  
          lectin co-stimulation can induce lymphocytes to grow in  
          serum-free medium. Recombinant human ILGF-I activity was  
          assayed as follows:  $1 \times 10^5$  lymphocytes were added to  
          individual wells of 96-well flat-bottomed tissue culture  
15    plates containing 100  $\mu$ l of RPMI-1640 tissue culture  
          medium, 5  $\mu$ g/ml PHA, 0.25% low endotoxin BSA and either  
          soluble rHuILGF-I or immobilized rHuILGF-I beads. The  
          mixture was incubated for 48 hours at 37°C. Next 1  
           $\mu$ Ci/well of [ $^3$ H]-thymidine was added and the mixture was  
20    incubated for another 18 hours. The cells were harvested  
          as described in Example 21. The results are summarized in  
          Table 18. They indicate that immobilized rHuILGF-1 on  
          polystyrene beads induce lymphocyte growth in a PHA  
          co-stimulation assay in serum-free medium.

25

Example 56Recombinant Human Insulin-Like Growth Factor II  
(rHuILGF-II) Immobilized on 9.63  $\mu$ m Blue-Dyed  
Polystyrene Beads Stimulates a Crude Lymphocyte  
Preparation to Proliferate in Serum-Free Medium

30           Recombinant human ILGF-II was immobilized on 9.63  
           $\mu$ m blue-dyed polystyrene beads as described in Example 41.  
          The immobilized rHuILGF-II beads were washed as described  
          in Example 21. The assay performed was as described in  
35    Example 55. Results are summarized in Table 19 and show  
          that immobilized rHuILGF-II beads induce lymphocyte growth  
          in a PHA co-stimulation assay in serum-free medium.



WO 90/09798

PCT/US90/01031

67

Example 57Immobilized Recombinant Human Tumor Necrosis Factor  
(TNF-alpha) Kills Murine LM Cells

5           Recombinant tumor necrosis factor alpha (TNF-alpha) immobilized on 9.64  $\mu$ m blue-dyed polystyrene beads kill murine LM cells in a 72 hour killing assay. Recombinant TNF-alpha was immobilized on 9.64  $\mu$ m blue-dyed polystyrene beads as described in Example 42. The

10 immobilized TNF-alpha beads were washed three times as described in Example 21. TNF-alpha killing was assayed using murine LM cells (American Type Culture Collection). The assay was as follows. Aliquots of either soluble TNF-alpha or immobilized TNF-alpha were added to individual

15 wells in a 96-well flat-bottomed tissue culture plate followed by the addition of  $1 \times 10^4$  LM cells. The mixtures were incubated for 72 hours. Killing was then assayed by either the addition of 1  $\mu$ Ci of [ $^3$ H]-thymidine to each well or MTT and the mixture incubated an additional 4 hours.

20 The thymidine-labeled cells were collected as described in Example 21. The MTT-labeled cells were fixed in isopropyl alcohol and the amount of MTT uptake measured by reading the absorbance at 590 nm. The results are reported in Table 20 and demonstrate that immobilized TNF-alpha

25 inhibits both thymidine uptake, MTT uptake, and oxidation, which indicates cell death.

Example 58Fibroblast Growth Factor Basic (FGFb) Immobilized  
on 2.85  $\mu$ m Polystyrene Beads Induces Growth of  
Murine 3T3 Cells in Growth Factor Depleted Medium

30           Immobilized FGFb stimulates growth of Murine 3T3 cells in growth factor depleted medium. Immobilized FGFb beads prepared according to Example 43 were washed three

35 times by suspension and centrifugation as described in Example 21. Murine 3T3 cells (American Type Culture Collection) were grown in 1,2-dimethoxyethane (DME) medium

WO 90/09798

PCT/US90/01031

68

with antibiotics and 10% calf serum (CS) as described by Gospodarowcz, Nature, 249, 123 (1974), incorporated herein by reference. 3T3 cells were isolated by trypsinization and plated at either 600 or 2000 cells/well (96-well plates) in DME medium plus 10% CS. 3T3 cells were incubated over night at 37°C. The next morning, the wells were washed three times, resuspended in DME medium containing 0.4% CS, and incubated an additional 24 hours to deplete the cells and medium of growth factors. After 24 hours in DME medium containing 0.4% CS, either soluble FGFb, immobilized FGFb, or 10% CS was added to individual wells and the 3T3 cells incubated an additional 24 to 48 hours. The cells were then labeled with 1  $\mu$ Ci/well of [<sup>3</sup>H]-thymidine and incubated for an additional 16 hours. The results are displayed in Table 21. They indicate that immobilized FGFb beads stimulated 3T3 cell growth to levels comparable to soluble FGFb.

#### Example 59

Transforming Growth Factor-beta-2 (TGF-beta-2)  
Immobilized on 2.85  $\mu$ m Polystyrene Beads Induces  
Growth of NRK-49F Cells in Growth Factor Depleted Medium

Immobilized TGF-beta-2 stimulates the growth of NRK-49F cells in growth factor depleted medium. Immobilized TGF-beta-2 beads prepared according to the method in Example 44 were washed three times by suspension and centrifugation as described in Example 21. NRK-49F cells (American Type Culture Collection) were grown in DME medium with antibiotics and 10% calf serum (CS) as described by Assoin et al., J. Biol. Chem., 258, 7155 (1973), incorporated herein by reference. NRK-49F cells were isolated by trypsinization and plated at a concentration of  $5 \times 10^3$  cells/well (96-well plates) in DME plus 10% CS medium. The cells were incubated over night at 37°C in 5% CO<sub>2</sub>, and then washed twice in DME medium containing 0.2% CS. The medium was replaced with 100  $\mu$ l DME plus 0.2% CS and the cells were incubated as above for

WO 90/09798

PCT/US90/01031

69

three to four days to deplete the medium of growth factors. When the NRK-49F cells had reached about 75% confluency, soluble TGF-beta-2a, immobilized TGF-beta-2a, or 10% CS was added to individual wells and the NRK-49F cells were incubated an additional 17 hours. Then 1  $\mu$ Ci of [ $^3$ H]-thymidine was added to the wells and the cells were incubated for another 4 hours before harvesting as described in Example 21. The results are listed in Table 22. They indicate that immobilized TGF-beta-2 beads stimulate NRK-49F cells to grow in growth factor depleted medium.

#### Example 60

##### Immobilized Recombinant Human Interferon-alpha-2a Kills the Interferon Sensitive HeLa S3 Cell Line

Immobilized recombinant Human Interferon-alpha-2A kills the interferon sensitive HeLa S3 cell line. Recombinant human Interferon-alpha-2a (INF-alpha-2a) immobilized on 2.85  $\mu$ m blue-dyed polystyrene beads inhibits [ $^3$ H]-thymidine uptake in a human epitheloid carcinoma cell line HeLa S3 (i.e., kills HeLa S3). Recombinant INF-alpha-2a was immobilized on 2.85  $\mu$ m blue-dyed polystyrene beads as described in Example 45. The immobilized INF-alpha-2a beads were washed three times as described in Example 21. INF-alpha-2a killing was assayed using a human epitheloid carcinoma cell line HeLa S3 (American Type Culture Collection). INF-alpha-2a blocks [ $^3$ H]-thymidine uptake which leads to cell death. The assay was as follows. Aliquots of either soluble INF-alpha-2a or immobilized INF-alpha-2a were added to individual wells in a 96-well flat-bottomed tissue culture plate followed by the addition of  $1 \times 10^4$  HeLa S3 cells. The beads with the INF-alpha-2a or soluble INF-alpha-2a were incubated for either 48, or 72 hours, at which time 1  $\mu$ Ci of [ $^3$ H]-thymidine was added to each well and the mixture incubated an additional 4 hours. The cells were collected as described in Example 21. The results are reported in

WO 90/09798

PCT/US90/01031

70

Table 23 and demonstrate that immobilized INF-alpha-2a inhibits thymidine uptake which leads to the death of the HeLa S3 tumor cells.

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Example 61

Recombinant Human Epidermal Growth Factor Immobilized  
on 0.93  $\mu$ m Polystyrene Beads Induces NRK-49F Cells  
to Grow in the Absence of Serum

Recombinant human epidermal growth factor  
10 (rHuEGF) immobilized on 0.93  $\mu$ m blue-dyed polystyrene  
beads induces NRK-49F cells to grow in the absence of  
serum. Recombinant human EGF was immobilized on 0.93  $\mu$ m  
blue-dyed polystyrene beads as described in Example 46.  
The immobilized rHuEGF beads were washed three times as  
15 described in Experiment 21. Serum contains many growth  
factors that are required by cells to grow in vitro. The  
assay procedure for the NRK-49F cells was as follows.  
NRK-49F cells were maintained in DMEM (Dulbecco's Modified  
Eagles Medium, Whittakar M.A. Bioproducts) medium plus 10%  
20 calf serum (CS). NRK-49F cells are plated at  $5 \times 10^3$  cells  
per well in 96-well flat-bottomed tissue culture plates  
and incubated for 24 hours in the 10% CS. The cells were  
then washed with serum-free medium and then replenished  
with serum-free DMEM. Aliquots of either soluble rHuEGF  
25 or immobilized rHuEGF were added to the individual wells.  
The beads with the rHuEGF or soluble rHuEGF were incubated  
for 24 or 48 hours. Growth was then measured by the  
addition of 1  $\mu$ Ci of [ $^3$ H]-thymidine to each well and the  
mixture was incubated an additional 6 hours. The cells  
30 were collected as described in Example 21. The results  
are reported in Table 24 and demonstrate that immobilized  
rHuEGF will induce murine NRK-49F cells to grow.

WO 90/09798

PCT/US90/01031

71

Example 62Recombinant Platelet-Derived Growth Factor Immobilized  
on 2.85  $\mu$ m Polystyrene Beads Induces Murine 3T3 Cells  
to Grow in the Absence of Serum

- 5           Recombinant platelet-derived growth factor (rHuPDGF) immobilized on 2.85  $\mu$ m blue-dyed polystyrene beads induce murine 3T3 cells to grow in the absence of serum. Recombinant human PDGF was immobilized on 2.87  $\mu$ m blue-dyed polystyrene beads as described in Example 47.
- 10   The immobilized rHuPDGF beads were washed three times as described in Experiment 21. Serum contains many growth factors that are required by cells to grow in vitro. Most cells will not grow if they are depleted of these growth factors. Murine Swiss 3T3 is such a cell line, which is
- 15   available from American Type Culture Collection. The assay procedure was as follows. Swiss 3T3 cells were maintained in DMEM medium plus 10% calf serum (CS). The 3T3 cells are plated at  $1 \times 10^4$  cells per well in 96-well flat-bottomed tissue culture plates and grown to
- 20   confluency. The medium was then changed to 2% CS, and the 3T3 cells remained viable but did not grow. Before growth factors were added, the cells were washed free of the 2% CS with serum-free DMEM, and then replenished with serum-free DMEM. Aliquots of either soluble rHuPDGF or
- 25   immobilized rHuPDGF were added to the individual wells. The cells were incubated for 16 hours. Growth was measured by the addition of 1  $\mu$ Ci of [ $^3$ H]-thymidine to each well and the mixture was incubated an additional 6 hours. The cells were collected as described in Example 21. The
- 30   results are reported in Table 25 and demonstrate that immobilized rHuPDGF will induce murine 3T3 cells to grow.

Example 63Growth of DA1-E5 Cells on Recombinant Human

- 35   Erythropoietin Immobilized on Co-Bind<sup>TM</sup> Polystyrene Plates
- Recombinant human erythropoietin (rHuEPO) immobilized on Co-Bind<sup>TM</sup> polystyrene plates induces growth

WO 90/09798

PCT/US90/01031

72

of EPO/IL-3 dependent DA1-E5 cells (see Example 54). Recombinant human EPO was immobilized on Co-Bind™ polystyrene plates as described in Example 48. Wells containing Immobilized rHuEPO were washed five times with 1X PBS, followed by washing five times with Iscove's MEM containing 10% heat-inactivated serum, then filled with 0.050 ml of IMDM with 10% serum. DA1-E5 cell growth was assayed as follows.  $1 \times 10^4$  cells were added to wells containing immobilized rHuEPO or soluble rHuEPO. The cells were incubated for 48 hours before either MTT or  $1 \mu\text{Ci}$  [ $^3\text{H}$ ]-thymidine was added to each well and the mixture was then incubated an additional 4 hours. The results are reported in Table 26 and demonstrate that immobilized rHuEPO will induce growth in EPO/IL-3 dependent DA1-E5 cells.

#### Example 64

##### Attachment of Recombinant Human Gamma-Interferon to Co-Bind™ Well Strips

Recombinant human gamma-interferon (rHuIFN-gamma) was obtained from Genzyme, Boston, MA, as a liquid formulation that contained  $1 \times 10^6$  U/ml ( $2.5 \times 10^7$  U/mg). An aliquot (0.02 mls,  $2 \times 10^4$  U) of this solution was diluted to 2.0 ml with PBS to give a stock solution that was  $1 \times 10^4$  U/ml. Four wells of the 8-well strip were then filled as shown below.

Well	<u>rHuIFN-gamma</u>		<u>PBS, mls</u>
	<u>mls</u>	<u>units</u>	
A	0.1	1000	0.1
B	0.05	500	0.15
C	0.01	100	0.19
D	0.005	50	0.195

After filling the wells, the strips were covered and incubated at  $37^\circ\text{C}$  for 3 hours, then processed exactly as described in Example 48. After washing thoroughly with PBS, the wells were filled with PBS, the strips were

WO 90/09798

PCT/US90/01031

73

covered and kept at 4°C until used.

#### Example 65

##### Biological Activity of Recombinant Human Gamma-Interferon

5 Human peripheral blood monocytes were isolated from blood drawn into a heparinized syringe and isolated by gradient centrifugation on 46% Percoll (Pharmacia, Newark, NJ). The monocytes were isolated from the interface, washed three times in phosphate buffered saline  
10 and resuspended in RPMI-1640 media containing 5% human AB sera to a concentration of  $1 \times 10^6$  cells per ml. Co-bind™ strips containing 4 wells gamma-interferon immobilized as in Example 67 were washed three times with phosphate buffered saline, washed three times with RPMI-1640 media  
15 containing 2% Fungi-Bact, and wiped with a sterile gauze. To each well was added  $1 \times 10^5$  monocytes in a volume of 0.1 ml. Soluble gamma-interferon was added to wells which did not contain the immobilized gamma-interferon. The cultures were incubated for 24 hours after which 0.1 ml of  
20 the media was removed and assayed for tumor necrosis factor production using commercially available Elisa kits. The results shown in Table 27 demonstrate that immobilized gamma-interferon is biologically active.

25 The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

WO 90/09798

PCT/US90/01031

74

Table 1

## Growth Factor Families

Family	Members
Epidermoid Growth Factors (EGF)	Epidermoid Growth Factor Transformation Growth Factor Alpha (TGF-alpha) Vaccinia Growth Factor (VGF) Shape Fibroma Growth Factor (SFGF) Myxoma Growth Factor (MGF) Amphiregulin (AR)
Platelet-Derived Growth Factor (PDGF)	PDGF-AA PDGF-AB PDGF-BB
Transformation Growth Factor Beta (TGF-beta)	TGF-beta 1 TGF-beta 2 TGF-beta 3 TGF-beta 4 Inhibins Mullerian Inhibiting Substance (MIS) Bone Morphogenic Proteins (BMP's)
Fibroblast Growth Factor (FGF)	Acidic FGF Basic FGF hst gene product int-2 gene product
Insulin-like Growth Factor (IGF)	IGF-I IGF-II Insulin Relaxin



WO 90/09798

PCT/US90/01031

75

Table 2  
Attachment of IL-2 to Polystyrene Blue-Dyed Beads:  
Effect of IL-2 Solution Concentration

	Beads 2.5% Suspension μl	PBS μl	IL-2 Solution μl	IL-2 Solution μg	Activity (units)* IL-2 Solution before after	% IL-2 Incorporated
5	125	250.0	0	0	-----	-----
	125	247.5	2.5	2.5	18,750	100.0
	125	245.0	5.0	5.0	37,500	100.0
10	125	240.0	10.0	10.0	75,000	97.8
	125	225.0	25.0	25.0	187,500	97.1
	125	200.0	50.0	50.0	375,000	92.8
	125	100.0	100.0	100.0	750,000	89.1
15	125	50.0	200.0	200.0	1,500,000	74.8
20					377,767	

\* Activity of the IL-2 solution before and recovered from supernatant after attachment of the IL-2 to the beads

WO 90/09798

PCT/US90/01031

Table 3  
Growth of CTLL-2 Cells Using Immobilized Recombinant IL-2  
(Ala-125 Analog)

5

Example No.	Recombinant IL-2 Type	<sup>3</sup> H]-Thymidine Incorporation (DPM's)	Concentration of IL-2 Bead (units/Beads)	Total IL-2 Activity (units/reaction)	Relative Activity Compared Soluble IL-2 (1000 units/ml)
1	Soluble IL-2 <sup>1</sup> (Control)	72,042 ± 9574	---	100 units	100%
2	9.64 μm Blue-dyed Beads <sup>3</sup> with Glutaraldehyde Linking Arm	90,975 ± 2855	0.030	3,000 units	126%
6	0.93 μm Blue-dyed Beads <sup>2</sup> with Glutaraldehyde Linking Arm	101,005 ± 8005	0.00046	4,600 units	140%
7	9.67 μm Carboxylate Beads <sup>3</sup> with 6-Aminocaproic Acid Linking Arm	32,708 ± 3108	0.027	2,700 units	45%
8	9.67 μm Carboxylate Beads <sup>3</sup> with 1,6-Diaminohexane/Glutaraldehyde Linking Arm	53,827 ± 8439	0.027	2,700 units	75%
9	9.67 μm Carboxylate Beads <sup>3</sup> with 1,12-Diaminododecane/Glutaraldehyde Linking Arm	66,319 ± 505	0.026	2,600 units	92%
		177,510 ± 14,175	0.047	4,720 units	246%

76

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Table 3 (continued)

5	---	Soluble IL-2 <sup>1</sup> (Control)	109,900 ± 10,550	---	100 units	100%
10	13	5.29 μm Amino Beads <sup>3</sup> with Glutaraldehyde Linking Arm	102,749 ± 10,007	0.026	2,600 units	93%
15	---	Soluble IL-2 <sup>1</sup> (Control)	134,190 ± 15,700	---	100 units	100%
20	10	65 μm Carboxylate Beads <sup>4</sup> with 1,12-Diaminododecane/ Glutaraldehyde Linking Arm	197,633 ± 1585	7.2	7,200 units	147%
25	---	Soluble IL-2 <sup>1</sup> (Control)	147,494 ± 32,083	---	100 units	100%
30	14	Sephadex <sup>®</sup> G-10 Beads <sup>5</sup> with 6-Aminocaproic Acid Linking Arm	195,328 ± 22,345	~45.0	9,000 units	132%
35	15	Sephadex <sup>®</sup> G-10 Beads <sup>5</sup> with 1,6-Diaminohexane Linking Arm	41,116 ± 976	~43.2	8,640 units	28%

1. IL-2 concentration was 1000 units/ml  
2. 1000 beads/cell starting concentration  
3. 10 beads/cell starting concentration (0.03 units IL-2/bead)  
4. 0.1 beads/cell starting concentration (7.2 units IL-2/bead)  
5. 10 μl of immobilized IL-2 Sephadex<sup>®</sup> G-10 beads/well (approximately 150-200 beads)

WO 90/09798

PCT/US90/01031

Table 4

## Growth of CRL-2 Cells Using Recombinant Natural Sequence IL-2

5

Example No.	Recombinant IL-2 Type	$^3\text{H}$ -Thymidine Incorporation (DPM's)	Concentration of IL-2 Bead (units/Beads)	Total IL-2 Activity (units/reaction)	Relative Activity Compared Soluble IL-2 (1000 units/ml)
10	---	---	---	---	---
15	Soluble IL-2 <sup>1</sup>	97,489 $\pm$ 9847	---	100 units	100%
20	IL-2 Immobilized on 9.64 $\mu\text{m}$ Blue-dyed Beads <sup>2</sup> with Glutaraldehyde Linking Arm	36,734 $\pm$ 3734	0.005	500 units	38%

78

1. Soluble IL-2 concentration was 1000 units/ml
2. 10 beads/cell starting concentration (0.005 units IL-2/bead)

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WO 90/09798

PCT/US90/01031

Table 5

Growth of CTL-2 Cells Using Immobilized Recombinant IL-2  
Polystyrene Beads:  
Carboxyl Group vs Amino Group Linkage

5

Example No.	Recombinant IL-2 Type	[ <sup>3</sup> H]-Thymidine Incorporation (DPM's)	Concentration of IL-2 Bead (units/heads)	Total IL-2 Activity (units/reaction)	Relative Activity Compared Soluble IL-2 (1000 units/ml)
10					
15					
20	--- Soluble IL-2 <sup>1</sup>	150,300	---	100 units	100%
	11 9.67 $\mu$ m Carboxylate Beads <sup>2</sup> with 1,12-Diaminododecane Linking Arm Attached to IL-2 via a Carboxyl Group	274,665	0.040	4,000 units	183%
25	1 9.63 $\mu$ m Blue-dyed Beads with Glutaraldehyde Linking Arm Attached <sup>3</sup> to IL-2 via an Amino Group	182,337	0.055	5,500 units	121%
30					

1. Soluble IL-2 concentration was 1000 units/ml
2. 10 beads/cell starting concentration (0.04 units/bead)
3. 10 beads/cell starting concentration (0.055 units/bead)

35

WO 90/09798

PCT/US90/01031

Table 6  
Growth of CTLL-2 Cells Using PEG-IL-2 Immobilized  
on Polystyrene Beads

Example No.	Recombinant IL-2 Type	[ <sup>3</sup> H]-Thymidine Incorporation (DPM's)	Concentration of IL-2 Bead (units/Beads)	Total IL-2 Activity (units/reaction)	Relative Activity Compared Soluble IL-2 (1000 units/ml)
---	Soluble IL-2 <sup>1</sup>	212,054 ± 19,556	---	100 units	100%
12	9.67 μm Carboxylate Beads with 1,12-Diaminododecane Linking Arm Attached to PEG-IL-2 via a Carboxyl Group	327,113 ± 14,379	0.023	2,300 units	154%
1. Soluble IL-2 concentration was 1000 units/ml 2. 10 beads/cell starting concentration (0.023 units/bead)					

WO 90/09798

PCT/US90/01031

Table 7

## CTIL-2 Growth On Recycled IL-2 Immobilized Beads

5

72 Hour Growth  
CycleIL-2 Immobilized Beads  
(3000 units IL-2)Soluble IL-2  
(1000 units IL-2)

10

	% of the Control	DPM's	sample control	% of the Control	DPM's	sample control
1	100%	10376		100%	12618	
2	93%	<u>13014</u> 13995		40%	<u>6498</u> 16330	
3 <sup>(2)</sup>	87%	<u>3160</u> 3628		8%	<u>565</u> 7344	81
4	79%	<u>15585</u> 19657		----	----	

15

20

- 25
1. Control is fresh IL-2 immobilized beads or soluble IL-2 set up at each new cycle
  2. All counts were low in cycle 3

WO 90/09798

PCT/US90/01031

82

Table 8

LAK Cell Activity Induced by Soluble Recombinant IL-2 or  
IL-2 Immobilized Beads

Cell Type	Treatment	K562	Lytic Units <sup>1</sup>	
			Raji	Daudi
Plastic Nonadherent Lymphocytes	None	6	0	0
	Soluble IL-2 (100 units/ml)	53	22	80
	IL-2 Immobilized Beads (1 bead/cell)	68	22	71
Total PBL's	None	4	0	0
	Soluble IL-2 (100 units/ml)	90	22	62
	IL-2 Immobilized Beads (1 bead/cell)	76	20	65

1. IUU (Lytic unit) = 20% killing with  $1 \times 10^6$  LAK cells



WO 90/09798

PCT/US90/01031

83

Table 9

NK/LAK Cell Killing of the Murine Target YAC-1 by in Vivo IL-2 Stimulated Lymphocytes from Spleen and Peritoneal Exudates

	Peritoneal Lymphocytes			
10	Effector Target Ratio	Control (200 µl PBS)	50,000 units Soluble IL-2 IL-2	9.64 µm beads 200,000 units 100,000 units IL-2
15			Percent Lysis	
20	2:1 5:1 10:1 25:1	1% 2% 2% 3%	1% 0% 2% 1%	2% 3% 3% 9%
	Spleen Lymphocytes			
25	Effector Target Ratio	Control (200 µl PBS)	50,000 units Soluble IL-2 IL-2	9.64 µm beads 200,000 units 100,000 units IL-2
30			Percent Lysis	
35	2:1 5:1 10:1 25:1	1% 1% 2% 5%	4% 5% 7% 16%	0% 0% 0% 4%

WO 90/09798

PCT/US90/01031

84

Table 10  
Growth of Crude T-Cells Using IL-4 Immobilized Beads  
and Suboptimal PHA Concentrations

	Donor 1		
	Experimental Conditions	[ <sup>3</sup> H]-Thymidine Incorporation (dpm's)	% of the Control
5			
10			
15	Nothing Added	338 ± 333	-----
	Soluble IL-4 (100 units/ml)	439 ± 263	-----
20	Control PHA (0.05 µg/ml)	54,831 ± 12,406	100%
	PHA (0.05 µg/ml) plus Soluble IL-4 (100 units/ml)	100,555 ± 30,822	183%
25	PHA (0.05 µg/ml) plus Soluble IL-4 (1 unit/ml)	65,127 ± 15,470	119%
30	PHA (0.05 µg/ml) plus Immobilized IL-4 (0.5 beads/cell)	59,602 ± 10,210	109%
35	PHA (0.05 µg/ml) plus Immobilized IL-4 (1 bead/cell)	67,350 ± 7,766	123%

WO 90/09798

PCT/US90/01031

85

Table 10 (continued)

5	Donor 2	Experimental Conditions	[ <sup>3</sup> H]-Thymidine Incorporation (DPM's)	% of the Control
10				
		Nothing Added	230 ± 231	----
15		Soluble IL-4 (100 units/ml)	385 ± 106	----
		Control PHA (0.05 µg/ml)	5,216 ± 1,215	100%
20		PHA (0.05 µg/ml) plus Soluble IL-4 (100 units/ml)	31,111 ± 5,552	596%
		PHA (0.05 µg/ml) plus Soluble IL-4 (1 unit/ml)	15,042 ± 4,653	288%
25		PHA (0.05 µg/ml) plus Immobilized IL-4 (0.5 beads/cell)	8,421 ± 1,627	161%
30		PHA (0.05 µg/ml) plus Immobilized IL-4 (1 bead/cell)	6,103 ± 2,201	117%

WO 90/09798

PCT/US90/01031

86

Table 11  
Growth of Crude T-Cells Using IL-6 Immobilized Beads  
and Suboptimal PHA Concentrations

Donor 1	Experimental Conditions	<sup>3</sup> H-Thymidine Incorporation (DPM's)	% of the Control
5			
10			
15	Nothing Added	351 ± 333	-----
	Soluble IL-6 (100 units/ml)	175 ± 263	-----
20	Control PHA (0.05 µg/ml)	55,385 ± 15,928	100%
	PHA (0.05 µg/ml) plus Soluble IL-6 (100 units/ml)	57,293 ± 19,551	103%
25	PHA (0.05 µg/ml) plus Soluble IL-6 (1 unit/ml)	41,163 ± 13,459	74%
30	PHA (0.05 µg/ml) plus Immobilized IL-6 (0.5 beads/cell)	98,996 ± 23,073	179%
35	PHA (0.05 µg/ml) plus Immobilized IL-6 (1 bead/cell)	98,865 ± 9,654	179%

WO 90/09798

PCT/US90/01031

87

Table II (continued)

5	Donor 2	Experimental Conditions	<sup>3</sup> H-Thymidine Incorporation (DPM's)	% of the Control
10				
15		Nothing Added	225 ± 220	----
		Soluble IL-6 (100 units/ml)	136 ± 57	----
		Control PHA (0.05 µg/ml)	5,022 ± 1,780	100%
20		PHA (0.05 µg/ml) plus Soluble IL-6 (100 units/ml)	6,163 ± 1,624	123%
25		PHA (0.05 µg/ml) plus Soluble IL-6 (1 unit/ml)	3,946 ± 808	79%
		PHA (0.05 µg/ml) plus Immobilized IL-6 (0.5 beads/cell)	33,074 ± 6,697	659%
30		PHA (0.05 µg/ml) plus Immobilized IL-6 (1 bead/cell)	37,408 ± 7,103	745%

WO 90/09798

PCT/US90/01031

88

Table 12  
Growth of AML-193 Cells on Immobilized Recombinant  
Human GMCSF Polystyrene Beads

Example No.	Recombinant Human GMCSF <sup>1</sup> (rHuGMCSF)	[ <sup>3</sup> H]-Thymidine Incorporation (DPM's)	% of the Control
---	Soluble GMCSF <sup>1</sup> (Control)	40,952 ± 1665	100%
33	rHuGMCSF Immobilized on 0.93 µm Blue-dyed Beads <sup>2</sup> with Glutaraldehyde Linking Arm	44,120 ± 9593	108%
33	rHuGMCSF Immobilized 0.93 µm Blue-dyed Beads <sup>3</sup> with Glutaraldehyde Linking Arm	51,586 ± 927	126%

1. Soluble recombinant human GMCSF concentration was 100 units/ml
2. 1.8 x 10<sup>3</sup> beads/cell initial concentration (6.67 x 10<sup>-6</sup> units/bead)
3. 1.8 x 10<sup>4</sup> beads/cell initial concentration

WO 90/09798

PCT/US90/01031

89

Table 13  
Growth of AML-193 Cells Using Immobilized Recombinant IL-3  
Polystyrene Beads

Example No.	Recombinant IL-3	[ <sup>3</sup> H]-Thymidine Incorporation (DPM's)	% of the Control
---	Soluble IL-3 <sup>1</sup> (Control)	20,517 ± 1169	100%
34	IL-3 Immobilized on 9.64 µm Blue-dyed Beads <sup>2</sup> with Glutaraldehyde Linking Arm	23,271 ± 1396	113%

1. Soluble IL-3 concentration was 1000 units/ml.  
2. 10 beads/cell starting concentration (0.15 units/bead)

WO 90/09798

PCT/US90/01031

90

Table 14

Growth of CTLH-2 Cells Using IL-2 Produced From LBRM.TG6 Cells  
Stimulated with Immobilized Recombinant IL-1-beta Polystyrene Beads

		MTT Incorporation Absorption at 590nm ( $A_{590}$ )	% of the Control
10	Control (10 $\mu$ g PUA/ml)	0.051 $\pm$ 0.003	0%
	Soluble IL-2 (10 units/ml)	0.122 $\pm$ 0.003	100%
15	Soluble IL-1-beta (10 units/ml)	0.069 $\pm$ 0.008	25%
	Soluble IL-1-beta (100 units/ml)	0.125 -----	104%
20	0.20 Beads/Cell (80 units Immobilized IL-1-beta)	0.098 $\pm$ 0.006	66%
	1.00 Beads/Cell (400 units Immobilized IL-1-beta)	0.123 $\pm$ 0.003	101%
25	1.00 Beads/Cell (Blank Beads containing BSA)	0.045 $\pm$ 0.001	0%

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WO 90/09798

PCT/US90/01031

91

Table 15

Growth of CTLI-2 Cells Using IL-2 Produced from LBRM.TG6 Cells  
Stimulated with Immobilized Human IL-1-alpha Polystyrene Beads

5	Conditions	MMP Incorporation Absorption 590 nm	% of the Control
10	Control	0.116 ± 0.009	0%
	<u>Soluble IL-1-alpha</u>		
15	10 units/ml	0.117 ± 0.007	13%
	100 units/ml	0.124 ± 0.014	100%
	<u>Immobilized IL-1-alpha</u>		
20	16 units/ml	0.130 ± 0.006	175%
	80 units/ml	0.209 ± 0.005	1163%
25	160 units/ml	0.174 ± 0.010	725%

Specific Activity = 0.0008 ng/lead

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WO 90/09798

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92

Table 16

Growth of AML-193 Cells Using Immobilized Recombinant Human GCSF  
(rHuGCSF) Polystyrene Beads

5	Conditions	<sup>3</sup> H]-Thymidine Incorporation (DPM'S)	% of the Control
10	<u>Soluble rHuGCSF</u>		
15	Control (none)	5713 ± 1702	0%
	10 units/ml	11000	60%
	50 units/ml	14525 ± 2595	100%
20	<u>Immobilized rHuGCSF Beads</u>		
	Control (no beads)	4552 ± 832	-13%
25	0.5 Beads/cell (10 units/ml)	7363 ± 1710	19%
	1.0 Beads/cell (20 units/ml)	8244 ± 2031	29%
30	2.0 Beads/cell (40 units/ml)	9503 ± 906	43%
	5.0 Beads/cell (100 units/cell)	10098 ± 1142	50%
35	10 Beads/cell (200 units/ml)	7398 ± 405	19%
40	Control - no beads or rHuGCSF added Specific Activity = 0.002 units/bead		

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93

Table 17  
Growth of DA1-E5 Cells on Covalent Versus Adsorbed rMuGMCSF  
Polystyrene Beads

Conditions	$^3\text{H}$ -Thymidine Incorporation (DPM's)	% of the Control
Control	2562 $\pm$ 1316	0%
<u>Soluble rMuGMCSF</u>		
100 units/ml	28758 $\pm$ 2976	100%
<u>Immobilized rMuGMCSF</u>		
Covalent rMuGMCSF	12278 $\pm$ 1544	37%
Adsorbed rMuGMCSF	464 $\pm$ 75	-8%
Blank BSA beads	2124 $\pm$ 132	-2%

Bead concentration was  $5 \times 10^6$  Beads/well for the covalent and adsorbed beads.

WO 90/09798

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94

Table 18

## Growth of Human Lymphocytes Using Immobilized rHuILGF-I Polystyrene Beads

	Donor 1		Donor 2	
	<sup>3</sup> H]-Thymidine Incorporation (DPM's)	% of the Control	<sup>3</sup> H]-Thymidine Incorporation (DPM's)	% of the Control
5				
10				
15				
	<u>Soluble rHuILGF-I</u>			
	Control (nothing)	0%	53481 ± 3074	0%
	10 ng/ml	100%	60590 ± 6405	81%
20	100 ng/ml	98%	62271 ± 4712	100%
	<u>Immobilized rHuILGF-I</u>			
	52 ng	72%	62353 ± 7067	101%
25	104 ng	54%	60814 ± 4120	83%

Specific Activity = 0.00052 ng/beads.

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Table 19

## Growth of Human Lymphocytes Using Immobilized rHuILGF-II Polystyrene Beads

5

	Conditions	Donor 1		Donor 2	
		<sup>3</sup> H]-Thymidine Incorporation (DPM's)	% of the Control	<sup>3</sup> H]-Thymidine Incorporation (DPM's)	% of the Control
10	<u>Soluble rHuILGF-II</u>				
	0	8444 ± 541	0%	19575 ± 1375	0%
	100 ng/ml	12327 ± 906	75%	21857 ± 1320	21%
20	200 ng/ml	13621 ± 443	100%	30431 ± 1421	100%
	<u>Immobilized rHuILGF-II</u>				
	42 ng/ml	11963 ± 1256	68%	27550 ± 1161	23%
25	84 ng/ml	13588 ± 485	99%	32091 ± 3193	115%

Specific Activity: 0.00042 ng/Bead.

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95

WO 90/09798

PCT/US90/01031

Table 20

## Killing of Murine LM Cells by Immobilized Recombinant Human TNF-alpha Polystyrene Beads

	Experiment 1	Experiment 2	Experiment 3
Conditions	Absorbance 590 nm (MTT uptake)	Absorbance 590 nm (MTT uptake)	Absorbance 590 nm (MTT uptake)
			[ <sup>3</sup> H]-Thymidine Incorporation (DPH's)
<u>Soluble TNF-alpha</u>			
Control	0.329 ± 0.045	0.358 ± 0.039	0.371 ± 0.083
10 units/ml	0.350 ± 0.003	0.466 ± 0.108	48283 ± 2472
100 units/ml	0.316 ± 0.039	0.436 ± 0.047	43621 ± 12828
1000 units/ml	0.097 ± 0.011	0.270 ± 0.004	11445 ± 13123
<u>Immobilized TNF-alpha</u>			
1 Bead/cell (384 units/ml)	0.205 ± 0.009	0.291 ± 0.024	0.305 ± 0.033
5 Beads/cell (1920 units/ml)	0.165 ± 0.011	0.168 ± 0.004	0.029 ± 0.001

56

WO 90/09798

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97

Table 21

Growth of Murine 3T3 Cells Using Immobilized FGfb Polystyrene Beads.

	Experiment 1		Experiment 2		
Growth Factor (concentration)	[ <sup>3</sup> H]-Thymidine Incorporation (DPM's)	% of the Control	[ <sup>3</sup> H]-Thymidine Incorporation (DPM's)	% of the Control	
10					
15					
	Control	2682 ± 1124	0%	3627 ± 1402	0%
	<u>Soluble FGfb</u>				
	1 ng	4145 ± 584	82%	-----	
20	5 ng	3489 ± 441	34%	-----	
	10 ng	2766 ± 810	5%	4276 ± 122	52%
25	50 ng	4474 ± 1115	100%	4880 ± 1261	100%
	<u>Immobilized FGfb</u>				
	18 ng	5546 ± 1230	160%	4172 ± 528	43%
30	36 ng	3184 ± 457	28%	2109 ± 162	0%
	10% CS	38544 ± 6699		67235 ± 10758	

Control - no growth factors added, just DME medium plus 0.4% CS  
 1. 600 3T3 cells/well; 24 hour exposure to FGfb before isotope was added.  
 2. 2000 3T3 cells/well; 24 hour exposure to FGfb before isotope was added.  
 3. 2000 3T3 cells/well; 48 hour exposure to FGfb before isotope was added.

Specific activity = 0.000114 ng FGfb/bead

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Table 21 (continued)

	Growth Factor (concentration)	Experiment 3		Experiment 4	
		[ <sup>3</sup> H]-Thymidine Incorporation (DPM's)	% of the Control	[ <sup>3</sup> H]-Thymidine Incorporation (DPM's)	% of the Control
5	Control	4123 ± 1602	0%	4846 ± 1095	0%
10	<u>Soluble FGFb</u>				
	1 ng	9747 ± 2295	59%	8114 ± 2522	54%
15	5 ng	11380 ± 4476	77%	10825 ± 1277	99%
20	10 ng	12275 ± 3496	86%	10871 ± 4152	100%
	50 ng	13600 ± 2045	100%	9069 ± 3247	70%
25	<u>Immoblized FGFb</u>				
	18 ng	19685 ± 3969	164%	6021 ± 507	20%
30	36 ng	10160 ± 912	64%	5224 ± 2109	6%
	10% CS	80946 ± 23421		104907 ± 3450	
35					

98

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Table 22

Growth of NRK-49F Cells Using Immobilized TGF-beta-2 Polystyrene Beads

5

	Experiment 1		Experiment 2		Experiment 3	
	Growth Factor (concentration)	[ <sup>3</sup> H]-Thymidine Incorporation (DPM's)	% of the Control	[ <sup>3</sup> H]-Thymidine Incorporation (DPM's)	% of the Control	[ <sup>3</sup> H]-Thymidine Incorporation Control (DPM's)
10	Control	14343 + 1859	0%	4725 + 891	0%	6000 + 1776
15	<u>Soluble TGF-beta-2</u>					
	0.5 ng	16286 + 2808	38%	-----	0%	4129 + 3058
20	1.0 ng	17704 + 3302	65%	4095 + 201	0%	6041 + 791
	<u>Immobilized TGF-beta-2</u>					
25	7.0 ng	13036 + 2740	0%	-----		11260 + 245
	18 ng	15749 + 3669	27%	5533 + 3557	30%	10301 + 1117
30	36 ng	22663 + 2731	162%	6092 + 1126	48%	8210 + 1976
	10% CS	19491 + 2001	100%	7520 + 942	100%	26828 + 4923

Control - no growth factors added, DME plus 0.2% CS.

35

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100

Table 23

Killing of HeLa S3 Tumor Cells by Immobilized Recombinant Human INF- $\alpha$ -2a  
Polystyrene Beads

5

	Conditions	48 Hour Exposure		72 Hour Exposure	
		[ <sup>3</sup> H]-Thymidine Incorporation (DPM's)	% Killing	[ <sup>3</sup> H]-Thymidine Incorporation (DPM's)	% Killing
10	<u>Soluble INF-<math>\alpha</math>-2a</u>				
	Control	114547 $\pm$ 12672	0%	124630 $\pm$ 25451	0%
	10 units/ml	56978 $\pm$ 10503	50%	73303 $\pm$ 27314	41%
20	100 units/ml	33000	71%	44062 $\pm$ 5708	65%
25	<u>Immobilized INF-<math>\alpha</math>-2a Beads</u>				
	24 Beads/cell (720 units/ml)	64411 $\pm$ 7375	44%	76756 $\pm$ 19220	38%
30	120 Beads/cell (7800 units/ml)	56237 $\pm$ 5652	51%	73735 $\pm$ 7160	41%

Specific Activity = 0.03 units/bead

35

WO 90/09798

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101

Table 24  
Growth of NRK-49F Cells Using Immobilized rhEGF  
Polystyrene Beads

	Conditions	Experiment 1 (24 hours)		Experiment 2 (48 hours)	
		[ <sup>3</sup> H]-Thymidine Incorporation (DPM's)	% of the Control	[ <sup>3</sup> H]-Thymidine Incorporation (DPM's)	% of the Control
5	<u>Soluble rhEGF</u>				
10	Control	1355 ± 313	0%	945 ± 828	0%
15	50 ng/ml	8252 ± 1709	100%	1922 ± 661	100%
20	100 ng/ml	7252 ± 1002	86%	1714 ± 572	79%
25	<u>Immobilized rhEGF</u>				
30	125 ng EGF	7352 ± 1070	87%	2275 ± 781	136%
35	625 ng EGF	7455 ± 1702	80%	1858 ± 239	93%
	<u>Serum Controls</u>				
	10% CS	16908 ± 1761		2985 ± 691	
	Specific Activity = 0.000025 ng/bead				

WO 90/09798

PCT/US90/01031

Table 25

Growth of Murine Swiss 3T3 Cells Using Immobilized PDGF  
Polystyrene Beads

5

	Conditions	Experiment 1		Experiment 2	
		[ <sup>3</sup> H]-Thymidine Incorporation (DPM's)	% of the Control	[ <sup>3</sup> H]-Thymidine Incorporation (DPM's)	% of the Control
10					
15	<u>Soluble rHuPDGF</u>				
	Control	1015 ± 71	0%	749 ± 778	0%
20	1 ng/ml	3996 ± 2143	34%	1506 ± 584	27%
	10 ng/ml	9795 ± 4128	100%	3847 ± 1559	100%
25	<u>Immobilized rHuPDGF</u>				
	150 ng PDGF	2877 ± 719	21%	1278 ± 2390	17%
	300 ng PDGF	9127 ± 903	92%	2575 ± 2390	59%
30	<u>Serum Controls</u>				
	2% CS	5860 ± 4351		1634 ± 1144	
35	10% CS	65230 ± 17090		26235 ± 3477	

Specific Activity = 0.0001 ng/bead

WO 90/09798

PCT/US90/01031

103

Table 26

**Growth of DA1-E5 Cells Using Immobilized Recombinant Human Erythropoietin  
(rHuEPO) Co-Bind™ Polystyrene Wells**

	Experiment 1		
	Conditions	[ <sup>3</sup> H]-Thymidine Incorporation (DPM's)	% of the Control
5			
10			
15	Control	305 ± 104	0%
	<u>Soluble rHuEPO</u>		
20	1 unit/ml	2363 ± 101	100%
	<u>Immobilized rHuEPO</u>		
	5 units/well	596 ± 465	14%
25	10 units/well	1013	34%
	20 units/well	1879	76%
30			

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104

Table 26 (continued)

5	Experiment 2	
	Conditions	Net Incorporation Absorbance at 590 nm
10	Control	0.100 0%
15	<u>Soluble rHuEPO</u>	
	1 unit/ml	0.280 100%
	5 units/ml	0.272 96%
20	<u>Immobilized rHuEPO</u>	
	50 units/well	0.274 97%
	100 units/well	0.219 66%
	200 units/well	0.295 108%
25		

WO 90/09798

PCT/US90/01031

105

Table 27  
Tumor Necrosis Factor Production (pg/ml)  
By Monocytes Exposed to Immobilized and Soluble Gamma-Interferon

5	Concentration <u>Gamma-Interferon</u>	<u>TNF Production (pg/ml)</u>	
		Immobilized	Soluble
10	control	2630	2630
	1 unit		5950
	10 units		8100
	50 units	4150	
	100 units	3600	12,500

WO 90/09798

PCT/US90/01031

106

## WHAT IS CLAIMED IS:

1. An immobilized cytokine comprising a cytokine bound to a solid support, said immobilized cytokine having  
5 substantially the biological activity demonstrated by the free cytokine, and wherein said immobilized cytokine is reusable.
2. The immobilized cytokine of claim 1 wherein said  
10 cytokine is covalently bound to said solid support.
3. The immobilized cytokine of claim 2 wherein said cytokine is covalently bound to said solid support using a urethane, triazine ether, amine, or amide linkage.  
15
4. The immobilized cytokine of claim 3 wherein said cytokine is covalently bound to said solid support using an amine or amide linkage.
- 20 5. The immobilized cytokine of claim 4 further including a linking arm wherein said cytokine is bound to said solid support by said linking arm and said linking arm comprises one or more linking groups selected from the group consisting of:  
25
  - (a) diamines, having the general formula  $\text{NH}_2\text{-R}^1\text{-NH}_2$ , where  $\text{R}^1$  is a  $\text{C}_2\text{-C}_{20}$  alkyl group;
  - (b) amino acids, having the general formula  $\text{NH}_2\text{-R}^2\text{-CO}_2\text{H}$ , where  $\text{R}^2$  is a  $\text{C}_1\text{-C}_{20}$  alkyl group;  
and
  - 30 (c) dialdehydes, having the general formula  $\text{OHC-R}^3\text{-CHO}$ , where  $\text{R}^3$  is a  $\text{C}_1\text{-C}_{20}$  alkyl group.
6. The immobilized cytokine of claim 5 wherein said linking arm comprises one or more linking groups  
35 selected from the group consisting of 6-aminocaproic acid, 1,6-diaminohexane, 1,12-diaminododecane, glutaraldehyde, and mixtures thereof.



WO 90/09798

PCT/US90/01031

107

7. The immobilized cytokine of claim 1 wherein said cytokine is selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, tumor necrosis factor, gamma-interferon, alpha-interferon, beta-interferon, erythropoietin, granulocyte colony stimulating factor, murine granulocyte colony stimulating factor, granulocyte-macrophage colony stimulating factor, murine granulocyte-macrophage colony stimulating factor, insulin-like growth factor I, insulin-like growth factor II, transformation growth factor beta, epidermoid growth factor, platelet derived growth factor, and fibroblast growth factor basic.
8. The immobilized cytokine of claim 7 wherein said cytokine is selected from the group consisting of IL-2, GMCSF, GCSF, EPO, TNF, FGFb, TGFb, PDGF.
9. The immobilized cytokine of claim 8 wherein said cytokine is a polyethylene glycol-modified IL-2 or an ala-125 IL-2 analogue.
10. The immobilized cytokine of claim 1 wherein said solid support is nonporous.
11. The immobilized cytokine of claim 10 wherein said solid support is a substantially spherical bead having a diameter of about 0.5-500  $\mu\text{m}$ .
12. The immobilized cytokine of claim 11 wherein said spherical bead has a diameter of about 1-75  $\mu\text{m}$ .
13. The immobilized cytokine of claim 12 wherein said solid support is a staple fiber having a diameter of about 5-200  $\mu\text{m}$ .

WO 90/09798

PCT/US90/01031

108

14. The immobilized cytokine of claim 10 wherein said support is selected from the group consisting of inorganic supports, including glass, quartz, ceramics, zeolites, metals, and metal oxides; polymeric materials including  
5 homopolymers, copolymers, or oligopolymers derived from monomeric units selected from the group consisting of styrene, divinylbenzene, ethylene, butadiene, acrylonitrile, acrylic acid, methacrylic acid, esters of acrylic and methacrylic acid, vinyl acetate, fluoro-  
10 alkenes, acrylamide and methacrylamide; carbohydrate supports, including agarose, cross-linked agarose, dextran, inulin, hyaluronic acid, cellulose, cellulose derivatives, starch and starch derivatives; and insoluble protein materials, including gelatin, collagen and  
15 albumin.

15. The immobilized cytokine of claim 14 wherein said support comprises a homopolymer, copolymer, or oligopolymer derived from monomeric units selected from  
20 the group consisting of styrene, divinylbenzene, ethylene, butadiene, acrylonitrile, acrylic acid, methacrylic acid, esters of acrylic and methacrylic acid, vinyl acetate, fluoroalkenes, acrylamide and methacrylamide.

25 16. The immobilized cytokine of claim 10 wherein said support includes a functionalized surface having a plurality of functional groups selected from the group consisting of hydroxyl, amino, carboxyl, sulfhydryl, and halogen.

30 17. A method for the in vitro growth of a cytokine dependent cell line comprising inducing growth of said cell line by contacting it with an effective amount of a cytokine bound to a solid support.

35 18. The method of claim 17 wherein said dependent cell line is CTTL-2 and said cytokine is IL-2.

WO 90/09798

PCT/US90/01031

109

19. The method of claim 17 wherein said dependent cell line is AML-193 and said cytokine is selected from the group consisting of HuGMCSF, HuGCSF, and IL-3.
- 5 20. The method of claim 17 wherein said dependent cell line is Balb/c 3T3 and said cytokine is PDGF or FGF-beta.
- 10 21. The method of claim 17 wherein said dependent cell line is NRK-49F and said cytokine is TFG-beta or EGF.
22. The method of claim 17 wherein dependent cell line is DA1-E5 and said cytokine is erythropoietin.
- 15 23. A method for the in vitro growth of cellular blood components comprising inducing growth of said components by contacting said components with an effective amount of a cytokine bound to a solid support.
- 20 24. The method of claim 23 wherein said cellular blood components are human peripheral blood lymphocytes.
- 25 25. A method for the in vitro growth of effector cells selected from the group consisting of lymphokine activated killer cells, natural killer cells, tumor infiltrating lymphocytes, and cytotoxic T-cells comprising inducing growth of said cells by contacting said cells with an effective amount of a cytokine bound to a solid support.
- 30 26. A method for the in vivo stimulation of the natural killer or lymphokine activated killer cells in the immune system of a host comprising injecting an effective amount of a cytokine bound to a solid support.
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WO 90/09798

PCT/US90/01031

110

27. A method for the in vivo stimulation of hematopoietic cell growth of a host comprising injecting an effective amount of a cytokine bound to a solid support.

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28. A method of claim 27 wherein said hematopoietic cells are granulocyte macrophages and said cytokine is GMCSF.

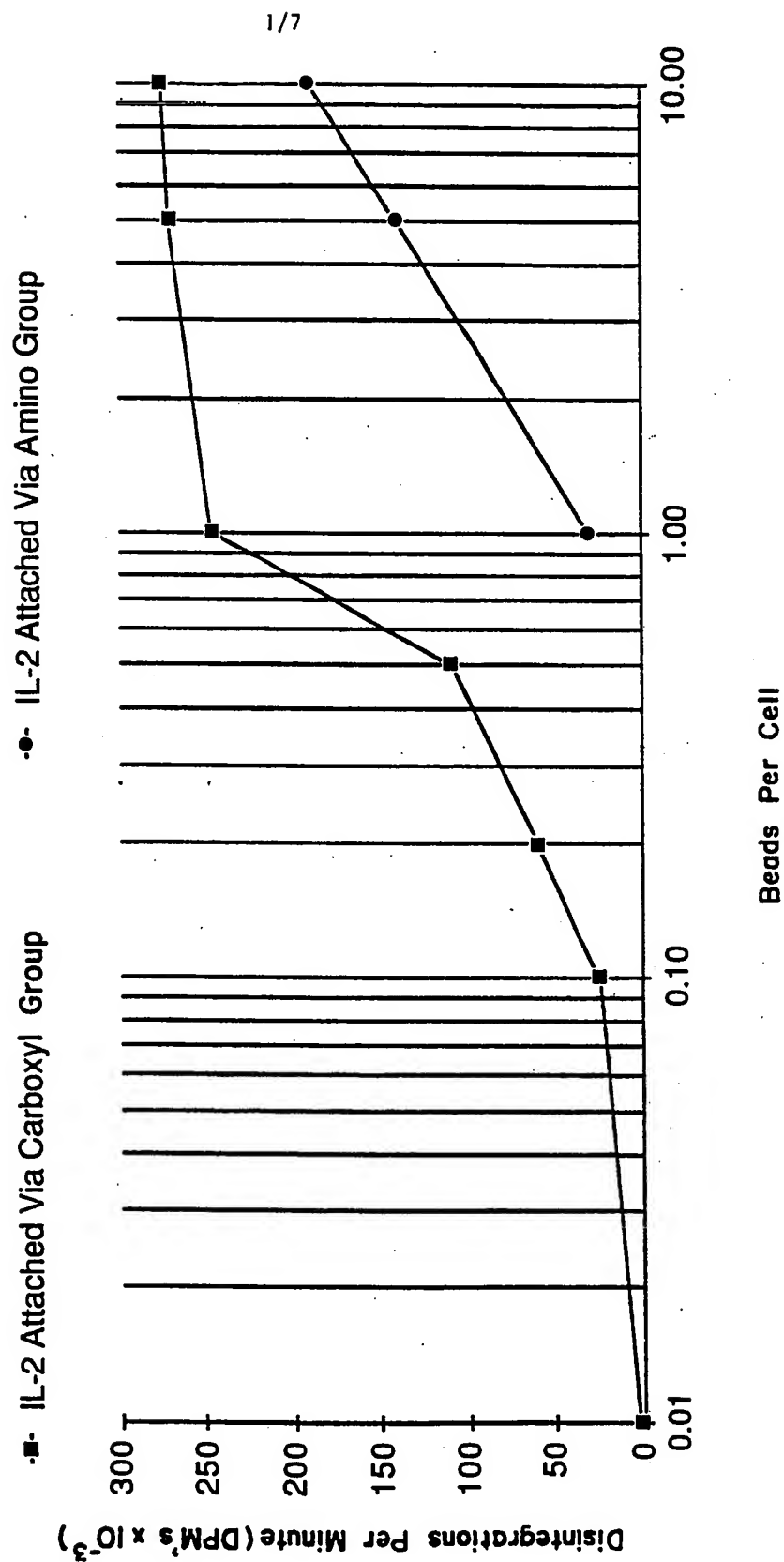
10 29. A method for the stabilization of a cytokine and substantially reducing proteolytic degregation in vivo, comprising attaching said cytokine to a solid support prior to introduction into the host.

15 30. A method for preventing the systemic absorption of cytokines, and the toxicity caused by the absorption of cytokines, comprising attaching said cytokine to a solid support, prior to introduction into the host.

WO 90/09798

PCT/US90/01031

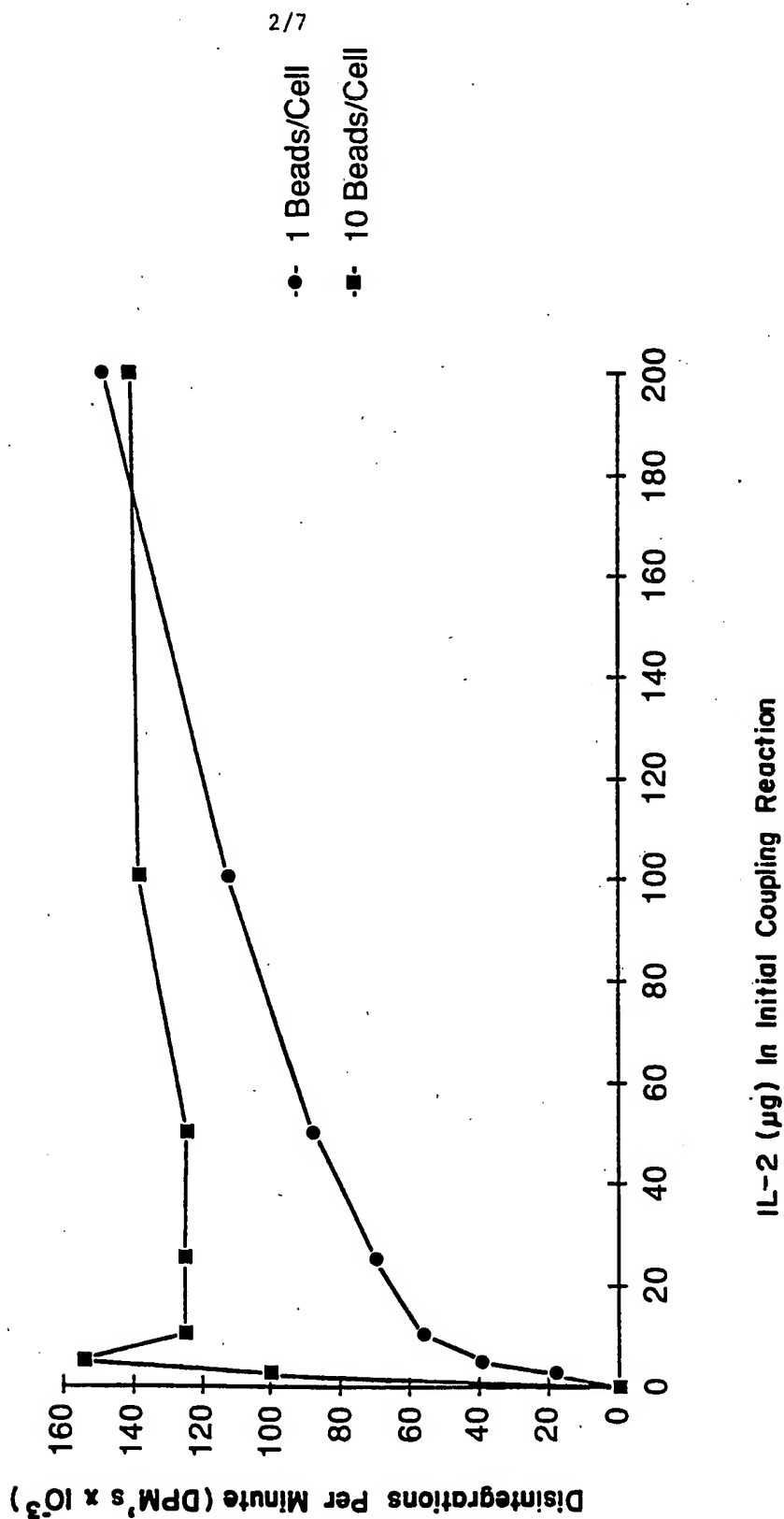
FIG. 1



WO 90/09798

PCT/US90/01031

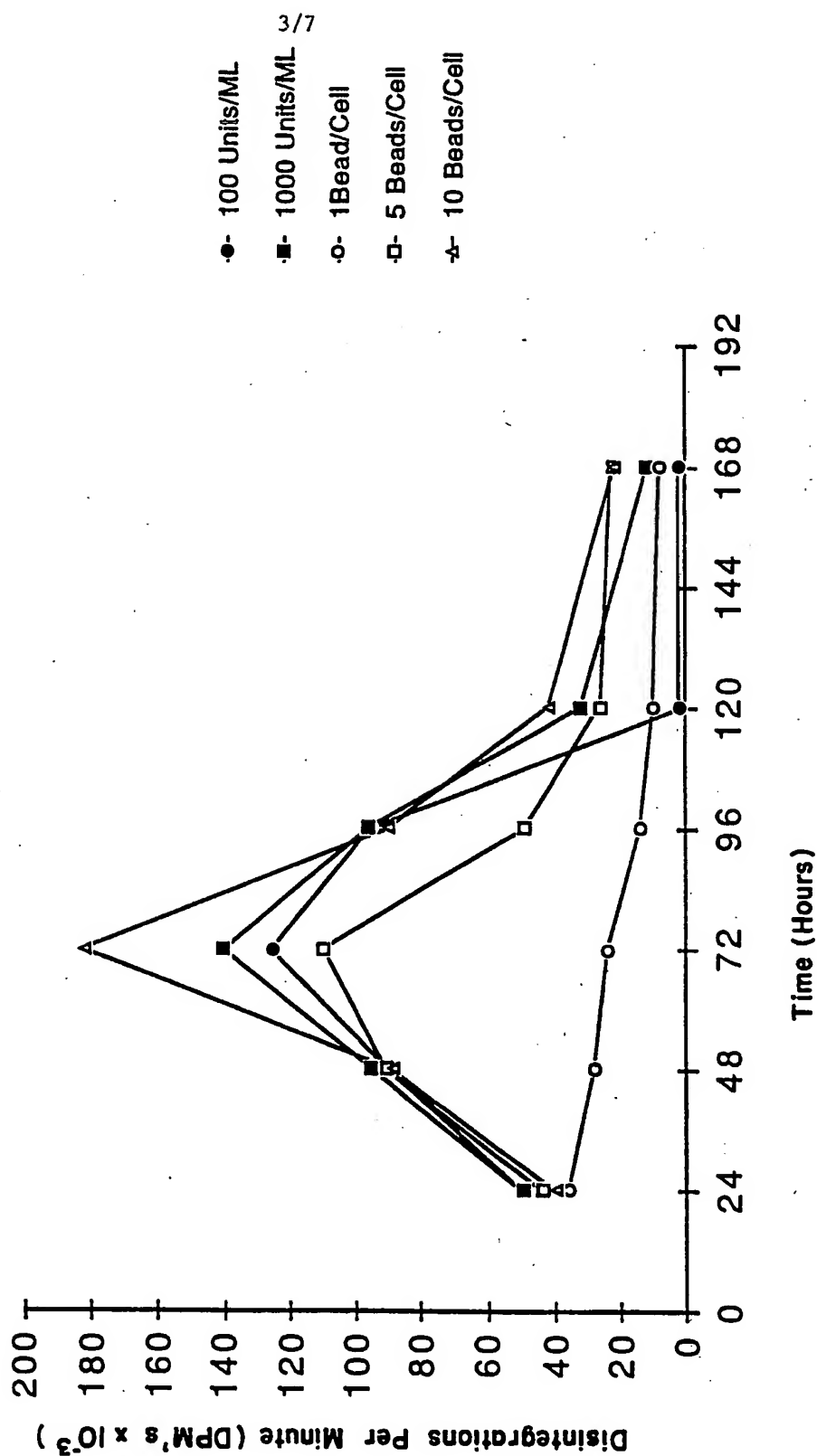
FIG. 2



WO 90/09798

PCT/US90/01031

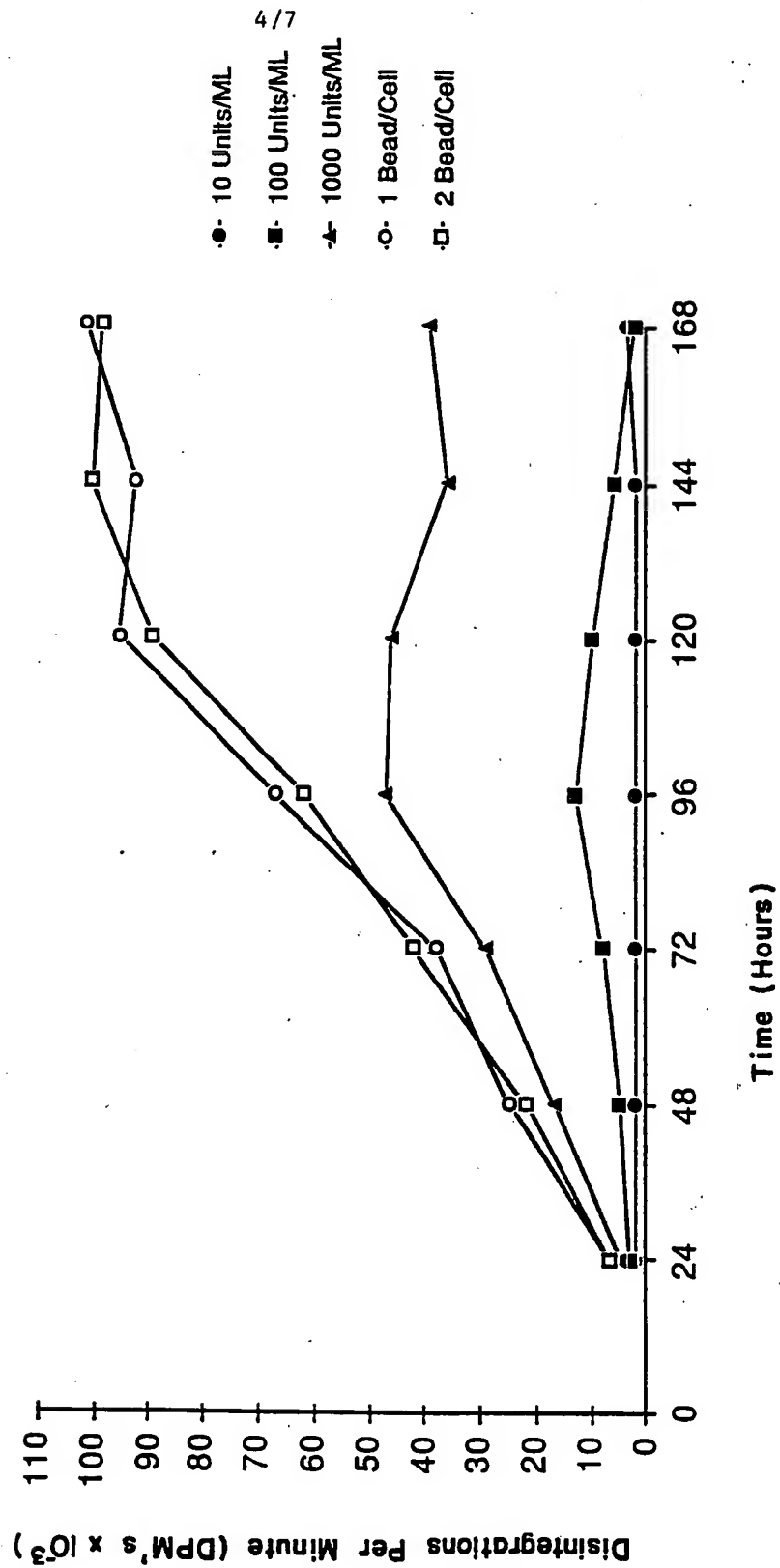
FIG. 3



WO 90/09798

PCT/US90/01031

FIG. 4





WO 90/09798

PCT/US90/01031

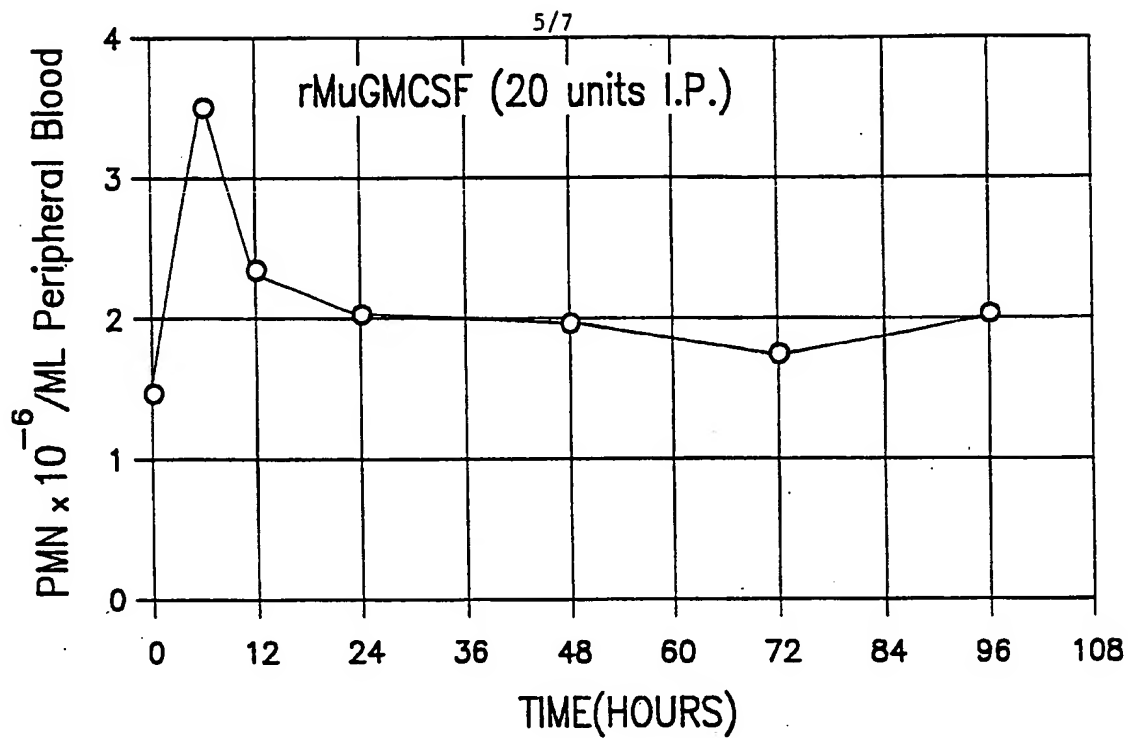


FIG. 5A

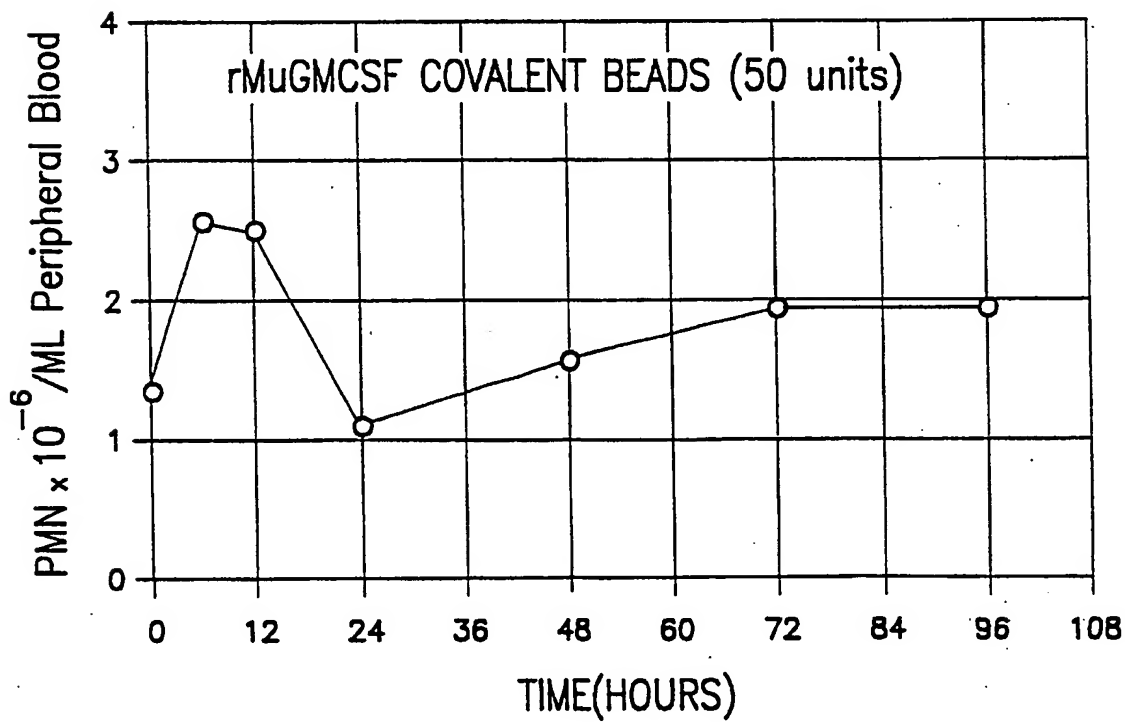


FIG. 5B

WO 90/09798

PCT/US90/01031

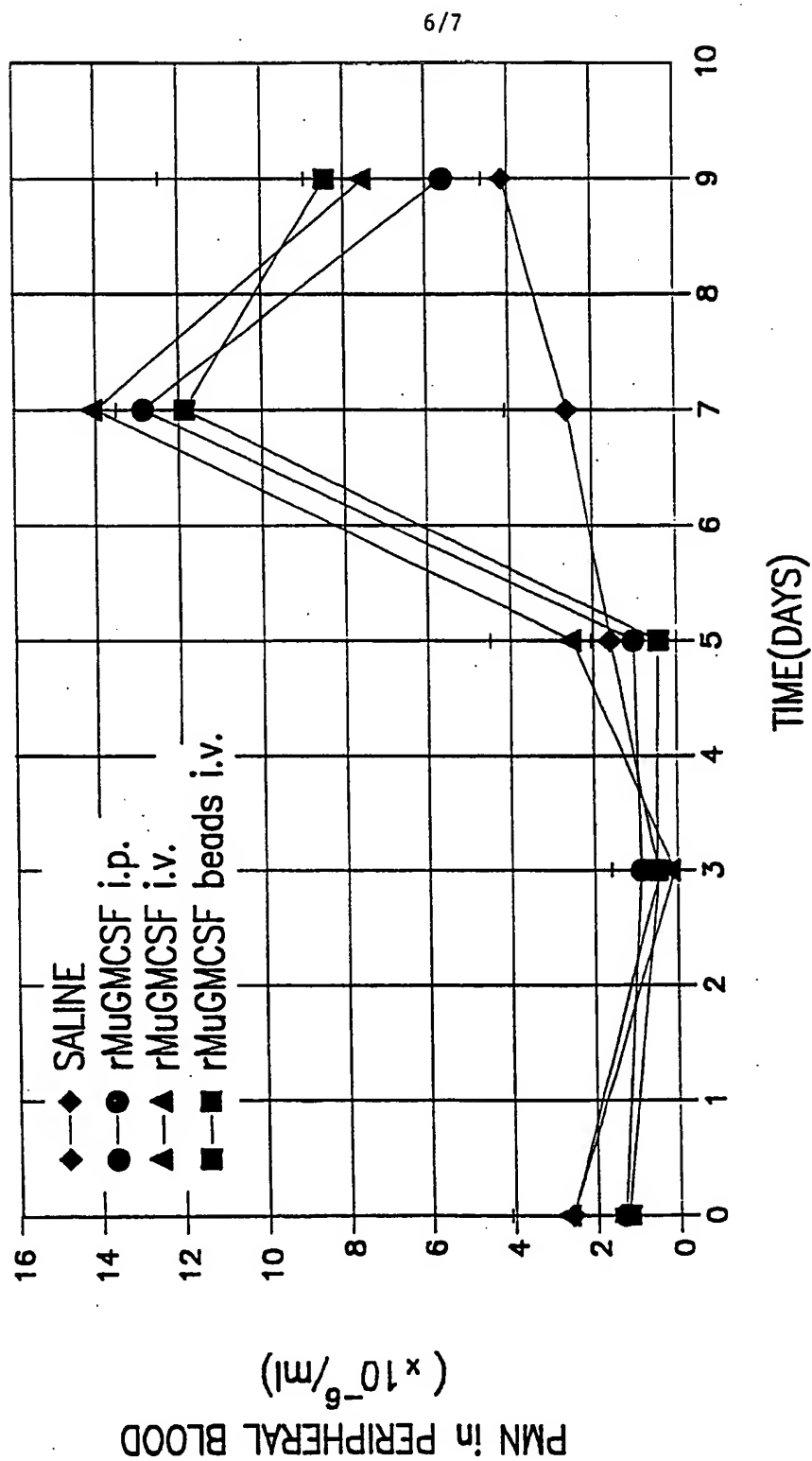


FIG. 6

WO 90/09798

PCT/US90/01031

7/7

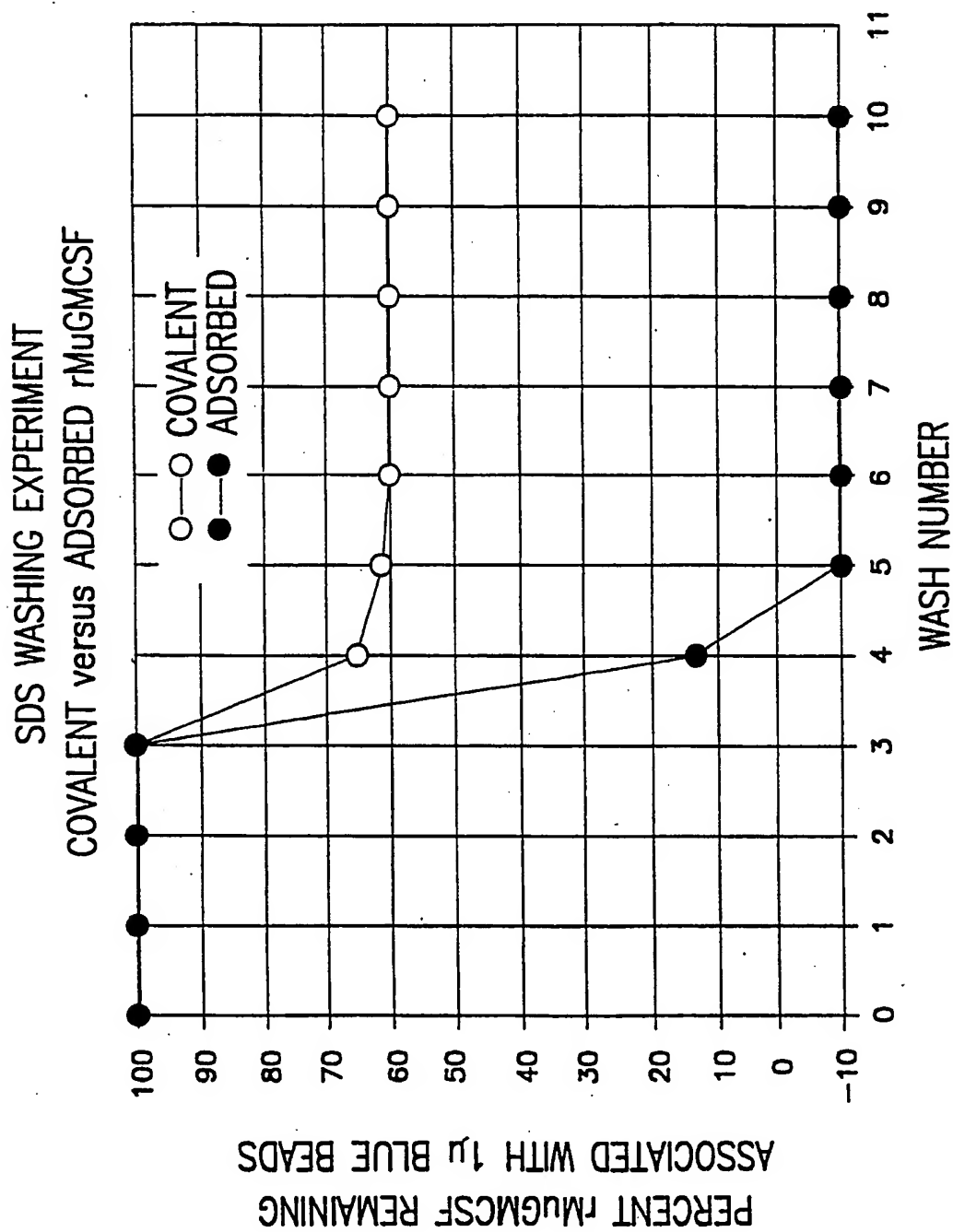


FIG. 7

## INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/01031

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) \*

According to International Patent Classification (IPC) or to prior art National Classification and 1979/14; C12N 5/00; (con't)  
 IPC(5): A61K 37/00, 37/10; C07K17/06, 17/08, 17/10, 17/14; C12N 5/00; (con't)  
 U.S. CL.: 514/2,8; 530/811,813,815,816; 435/240.2; 424/85.1 (con't)

## II. FIELDS SEARCHED

Minimum Documentation Searched \*

Classification System	Classification Symbols
U.S.	514/2,8; 530/810,811,812,813,814,815,816; 435/174,177,178,180,181,240.1,240.2,240.21, 240.51; 424/78,85.1,85.2,85.4,85.5,85.6,85.7
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched *	

## III. DOCUMENTS CONSIDERED TO BE RELEVANT \*

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages †	Relevant to Claim No. ‡
X Y	JP,A, 61-53,300 (KATO et al). 17 MARCH 1986, See entire document.	1-10,14-16 11-13,17-30
X Y	US,A, 4,609,546 (HIRATANI) 02 SEPTEMBER 1986, See entire document	1-4,7-10,14-16 5,6,11-13,17-30
Y	US,A, 3,639,213 (GINGER et al) 01 FEBRUARY 1972, See entire document	11-13
Y	US,A, 4,240,662 (RECKEL et al) 20 FEBRUARY 1979, See entire document	11-13
Y	US, A, 4,764,466 (SUYAMA et al) 16 AUGUST 1988, See entire document	11-13

## \* Special categories of cited documents: †

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Δ" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

21 MAY 1990

Date of Mailing of this International Search Report

11 JUL 1990

International Searching Authority

ISA/US

Signature of Authorizing Officer

DAVID M. MATT

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CON'T OF CLASSIFICATION OF SUBJECT MATTER

IPC(5): A61K 45/05, 37/66

U.S. CL: 424/85.2,85.4,85.5,85.6,85.7